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(54) Title: METHOD FOR IDENTIFYING MEMBERS OF COMBINATORIAL LIBRARIES

(57) Abstract

A method to determine the molecular weights of femtomole or smaller quantities of small peptides, oligonucleotides, or heterocyclics covalently attached to addressable polystyrene beads on a grid is presented using imaging time-of-flight secondary ion mass spectrometry (TOF-SIMS). The determination is made possible by selectively clipping the bond linking the peptide, oligonucleotide, or heterocyclic to the bead, followed directly by a TOF-SIMS assay of the bead on the grid. The method can be applied to large numbers of 10-120 micron polystyrene beads having different small molecules attached thereto for direct characterization of massive combinatorial libraries.

METHOD FOR IDENTIFYING MEMBERS
OF COMBINATORIAL LIBRARIES

CROSS-REFERENCE TO RELATED APPLICATION

This is a continuation-in-part application of copending U.S. patent application Serial No. 08/217,046, filed March 23, 1994.

FIELD OF THE INVENTION

The present invention relates to a method for the
5   identification and analysis of members of combinatorial libraries, wherein the identified member has a demonstrated pharmacological or physiological activity.

BACKGROUND OF THE INVENTION

10   Over the past ten years, there has been a growing demand for the production and identification of small molecules that have pharmacological activity as, for example, agonists or antagonists of various cellular acceptor molecules, such as cell-surface receptors,
15   enzymes, or antibodies. Such small molecules can be peptides, oligonucleotides, or other organic compounds, such as heterocyclics and the like. The unifying feature of these small molecules is operational in that they bind specifically to known acceptors. In consequence of such
20   binding, a physiological response occurs whereby certain biological processes are modulated, which can have applications in medicine and agriculture.

Searching for small molecules that are useful as pharmaceuticals entails (1) generating collections of
25   such molecules, (2) screening such molecules for physiological activity, and (3) identifying the structure of molecules that provide a positive result in the screen. The first two steps can be accomplished using methods well-known in the art, some of which are

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described herein for purposes of clarity. The third
step, where one determines the structure of a positively
screened small molecule, has proven to be the time-
limiting step in the overall process to discover new
5   small molecule pharmaceuticals. This step is necessary
to eliminate false positives or duplicates, and, of most
importance, to produce the selected small molecule for a
prospective pharmaceutical formulation.

Searching for such small molecules has involved
10   screening collections of natural materials, such as
fermentation products, plant or animal tissue extracts,
or libraries of synthesized molecules. Chemical assays
have been designed that merely identify those species
that bind a particular acceptor molecule or, in a
15   bioassay, assess the ability of tested molecules to
effect certain physiological reactions. Screening of
such collections often, at most, provides leads that must
be refined by more stringent techniques and expanded
testing of related molecules. All of these techniques
20   are limited severely by the available concentration of
any particular small molecule and the resolving power of
the screening and analysis techniques. As a result, the
process of production and identification of small
molecules that have pharmacological activity, a process
25   termed "irrational drug design" by Brenner and Lerner
(Proc. Natl. Acad. Sci. USA, 89, 5381-5383 (1992)),
"requires continual improvement both in the generation of
repertoires [of small molecules] and in the methods of
selection." Id. at page 5381.
30   A repertoire of small molecules, wherein each
molecule thereof can be represented preferably in at
least femtomole quantities, typically is produced by what
are termed multiple methods of synthesis or parallel
chemical synthetic protocols. Such repertoires are
35   commonly referred to as "combinatorial libraries," for
reasons that will become plain below. With reference to

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peptides, such synthetic methods have been disclosed by
Jung and Beck-Sickinger (Angew. Chem. Int. Ed. Engl., 31,
367-383 (1992)). Methods for the production of
heterocyclic libraries (see Bunin and Ellman, J. Am.
5   Chem. Soc., 114, 10997-10998 (1992)) and nucleic acid
libraries (referred to in Brenner and Lerner, supra) have
also been published. Other methods for the construction
of combinatorial libraries include those of Kerr et al.,
J. Am. Chem. Soc., 115, 2529 (1993); Lam et al., Nature,
10   354, 82 (1991); Houghten et al., Nature, 354, 84 (1991);
and Fodor et al., Science, 251, 767-773 (1991) (see, also
U.S. Patent No. 5,143,854 (1992)).

        In the methods cited above, members of a library are
constructed from the coupling of chemical building
15   blocks, such as amino acids, nucleic acids, or variant
organic monomers and side groups. Resultant libraries
consist of different individual species, the potential
number (k) of which can be calculated as a function of
the number of different building blocks used (a) and the
20   number of different building blocks coupled to each
member of the library (b), according to the following
formula: $k = a^b$. Thus, a library of pentapeptides
constructed using 20 different amino acids (i.e., the
chemical building blocks) could include as many as 20^5 or
25   3.2 million different species.

        The method of Lam et al., supra, is presented as an
example of one such method that provides a means to at
least approach the theoretical maximum number of
different species in a combinatorial library. The Lam et
30   al. method employs a "split synthesis" protocol, in which
standard solid phase peptide synthesis (see, e.g.,
Atherton and Sheppard, Solid Phase Peptide Synthesis, A
Practical Approach (Oxford University Press, 1989)) is
conducted on resin beads. Separate reactions for each
35   amino acid used take place to couple covalently one amino
acid to an aliquot of resin beads. For example, 20

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different reaction vessels may be used, in which the
resin beads are coupled to one of the 20 naturally
occurring proteinogenic amino acids. Typically, the
amino acids used in such reactions have been modified
5   using suitable blocking groups known in the art to allow
the coupling of only one amino acid per bead. After a
first reaction, the aliquots of resin beads having
attached thereto different single amino acids are
combined, thus completing the first round. A second
10   round to create dipeptides begins by removing the
blocking group from the last amino acid added, re-
allocating aliquots of the resin beads into another 20
reaction vessels, and allowing thereby the coupling of a
second single amino acid to each resin bead. The
15. combining of the resin beads having dipeptides completes
round two. The rounds are repeated until the library of
peptides has attained the desired number of building
blocks, which, in this case, are amino acids.

        According to the Lam et al. reference, each resin
20   bead processed as recited above contained about 50 to 200
picomoles of peptide, which presumably each consisted of
five amino acids. The library can then be screened for
those beads that include peptides that are recognized by
a particular acceptor molecule that is labelled directly
25   or indirectly with fluorescein or an enzyme, for example,
using materials and methods that are well known in the
art. Such a labelled bead may be isolated physically
using micromanipulation techniques, or its location,
i.e., address, may be noted for further analysis in situ,
30   i.e., in the midst of the nonselected, unlabelled beads
of the library. An alternate approach, proposed by
Brenner and Lerner, supra, would include an "appended
'genetic' tag" that would be interpreted to provide the
structure of each molecular species in a library;
35   however, this approach requires that the genetic tag be
added chemically to the individual molecular species,

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which could interfere with the ability of a molecular
species to interact with the acceptor molecule of
interest. Even if the genetic tag presented no such
obstacle, such tagged molecular species also would have
5   to be "read" in the midst of multiples of the non-
selected species. The current methods, in essence, have
not overcome adequately the challenge presented in either
isolating a labelled microscopic bead in view of the
large numbers that require analysis (discussed further
10   below) or in readily analyzing the identity of a
molecular species attached to a labelled bead when
surrounded by identical, unlabelled beads having
different molecular species attached to them.
          Presuming that the bead of interest can be isolated
15   physically, the contained peptide may be analyzed for its
sequence of amino acids using a commercially available
peptide microsequencer, such as Model 477A of Applied
Biosystems, for example. According to Lam et al.,
although "[a] library containing several million beads
20   could be screened [with labelled acceptor molecules] in
10-15 Petri dishes in an afternoon[, only about] ...
three pentapeptide beads were sequenced daily using the
microsequencer." Evidently, as understood from the
technical literature presented hereinabove, the limiting
25   step in the process of identifying new drugs from
combinatorial libraries is the step of discarding false
positives and determining the identity of the species of
interest, which difficulty includes the step of either
isolating the labelled bead(s) from unlabelled beads or
30   having a sufficiently discerning technology available
that can analyze the molecular species on a microscopic
bead when adjacent to identical beads having different
molecular species attached thereto.
          In the instance of identifying a peptide of
35   interest, for example, the time limiting step of
extracting the sequence of those binding peptides,

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however, is also limitative in that only peptides
containing naturally occurring amino acids can be
identified. This limitation is due to characteristics of
the Edman degradation technology upon which
5 microsequencers are based. In addition to having the
capability to sequence only a few peptides per day,
microsequencers can only sequence peptides that include
naturally-occurring proteinogenic amino acids.

       Accordingly, the analysis of any combinatorial
10 library is necessarily impeded by the very low rate at
which, in the Lam et al.-type method, beads having
members of the library attached thereto can be analyzed
for the identity of the attached molecule. In view of
the literally millions of candidate molecules to be
15 screened in a given library, it is probable that at least
hundreds, if not thousands, of the molecular species-
attached beads would generate positive signals (including
false positive signals) requiring further analysis. The
limitation of being able to sequence only a few molecules
20 per day, therefore, presents a strong drawback to current
strategies of screening combinatorial libraries for
pharmaceutical compounds. Moreover, if a method allowed
analysis of a positively signalled bead having a small
molecule of interest attached thereto without having to
25 remove such a bead from the group of other beads, in the
presence of which the bead was screened, the procedure of
screening and identifying small molecules of interest
would be greatly improved.

30   SUMMARY OF THE INVENTION

       It has now been discovered that a direct mass
spectrometric assay can be configured to read a wide
variety of combinatorial libraries, including those
composed of peptides, oligonucleotides, and heterocyclic
35 molecules. Using the present invention, any
combinatorial library can be constructed on a suitable

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substrate and screened, and the individual substrate that
is identified as having a molecule that specifically
interacts with an acceptor molecule of interest (i.e.,
positive screen result) can be identified in the presence
5 of identical substrates having other unselected molecular
species attached thereto and subjected to direct mass
spectrometric assay without removal from the total
library to determine the precise molecular weight of the
selected molecule. A preferred aspect of the method
10 includes the use of novel linking moieties or substrates
having reactive groups attached thereto that covalently
or ionically link the individual molecules of the
combinatorial library to the substrate, whereby the
linkage may be broken without disturbing the molecule's
15 structure, yet allow the library molecules to remain in
close association with the substrate based on physical
effects. Consequently, the present invention greatly
improves the ability of artisans of the relevant art to
identify pharmaceutically active agents derived from
20 combinatorial libraries.
        Accordingly, the present invention relates to a
method of identifying individual small molecules of a
combinatorial library comprising (a) forming a plurality
of complexes of solid substrates and the small molecules,
25 each of which comprises one substrate, or portion
thereof, and one of the small molecules of said library;
and (b) determining the molecular weight of a selected
small molecules by means of secondary ion mass
spectrometry. Suitable linking moieties and substrates
30 having suitable reactive groups attached thereto that
connect the small molecules to the substrate are
disclosed as well.
        These and other features and advantages of the
invention will be more readily apparent upon reading the
35 following detailed description of the invention and upon
reference to the accompanying drawings, all of which are

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given by way of illustration only, and are not limitative of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

5   Figure 1 is a schematic diagram of the imaging time-of-flight secondary ion mass spectrometry (TOF-SIMS) apparatus.

Figure 2 is a composite of three related mass spectrum profiles of phenylalanine attached to a
10   polystyrene bead by various means, as follows: by physical adsorption (Figure 2A); by covalent bonding (Figure 2B); and by physical adsorption after vapor phase clipping with trifluoroacetic acid (TFA) of linking covalent bond(s) (Figure 2C).

15   Figure 3 is a profile of a tripeptide associated with a polystyrene bead by physical adsorption only and placed on a copper grid.

Figure 4 is a composite of two TOF-SIMS images, each directed at the same address on a copper grid. Figures
20   4A and 4B display the $(M+H)^+$ ion intensity for phenylalanine and copper, respectively.

Figure 5 is a composite of two TOF-SIMS images, each directed at the same address on a copper grid. Figures 5A and 5B display the $(M+H)^+$ ion intensity for leucine
25   and phenylalanine, respectively.

Figure 6 is a profile of a tripeptide that was covalently linked to a polystyrene bead using an acid vapor labile linkage and then exposed to acid vapor, and then placed on a copper grid. Included with Figure 6 is
30   a representation of the structure of the tripeptide V-Y-V marked to indicate the fragments identified in the TOF-SIMS profile.

Figure 7 depicts the linking moieties attaching the angiotensin II receptor antagonist to various polystyrene
35   beads.

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Figure 8 is the composite of two electrospray mass spectra of the angiotensin II receptor antagonist. This data was provided for comparison with the present invention by S.A. Carr, M.E. Hemling, G.D. Roberts, and
5   J. Weinstock of the Chemical and Biological Research Division of SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania. Figure 8A displays the standard electrospray mass spectrum and Figure 8B displays the electrospray MS/MS spectrum.

10   Figure 9 is the composite of matrix assisted laser desorption (MALDI) spectra of angiotensin II receptor antagonist. This data was provided for comparison with the present invention by S.A. Carr, M.E. Hemling, G.D. Roberts, and J. Weinstock of the Chemical and Biological Research Division of SmithKline Beecham Pharmaceuticals,
15   King of Prussia, Pennsylvania. Figure 9A displays the standard MALDI spectrum and Figure 9B displays the post source decay spectrum.

Figure 10 is the TOF-SIMS mass spectrum of
20   angiotensin II receptor antagonist on a Sasrin bead after cleavage by TFA/CH_2Cl_2 vapors.

Figure 11 is the composite of two images of the angiotensin II receptor antagonist. Figure 11A displays the image of the $(M+H)^+$ ion (m/z 453.2) and Figure 11B
25   displays the image of the fragment ion (m/z 135).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following detailed description of the preferred
30   embodiments of the instant invention is provided to aid those skilled in the art in practicing the present invention, but should not be construed to limit the present invention, as modifications and variations in the embodiments herein discussed may be made by those of
35   ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

10

The present invention provides a method and novel
materials used in the inventive method that greatly
improves the ability of an ordinary artisan to identify
and characterize pharmaceutically-active small molecules
5 selected from a combinatorial library. The members of
such a library preferably are constructed in association
with a suitable substrate, such as a polystyrene bead
surface. Such association between the small molecules
and the substrates may be mediated by any suitable means,
10 including, but not limited to, physical adsorption,
covalent linkage, ionic bond, hydrophobic interactions,
and van der Waals forces. Preferably, such associations
are mediated by covalent or ionic linkage during the
construction of such combinatorial libraries, wherein
15 such a covalent or ionic linkage may be broken using
means that does not modify or substantially modify the
structure of the linked small molecule, and wherein the
small molecule remains in association with the substrate
via physical adsorption or other effects, but will allow
20 desorption in a secondary ion mass spectrometry (SIMS)
apparatus. Construction of such a library was described
above in the Background section, using the method of Lam
et al. (supra), as an example. Screening of such a
library was also described above in the Background
25 section. The present invention relates to the
identification of a positively screened small molecule
derived from the aforementioned combinatorial library.

A preferred identification approach would take into
account the fact that small molecules, such as peptides,
30 oligonucleotides, or heterocyclic compounds, may be
constructed such that they can be desorbed intact or
substantially intact from a substrate, particularly from
a bead surface. Because each bead, for example, may have
adsorbed thereto only a femtomole quantity of a
35 particular molecular species, or less, extreme
sensitivity of the method of analysis is required. For

example, a standard 40 micron sphere covered with one
layer of phenylalanine will only have about 50 femtomoles
of surface molecules available for sampling.

The present method directly assesses the molecular
5   weight of such molecular species upon their removal from
the substrate and immediate subsequent ionization. The
method employs imaging secondary ion mass spectrometry to
identify the molecular weights of molecules adsorbed to
the polystyrene bead surfaces, such as magnetic sector
10   SIMS, quadrupole SIMS, Fourier Transformation SIMS, or
time-of-flight SIMS (TOF-SIMS). The methodology actually
used for any given SIMS analysis is known in the art, and
may vary both with the machine used and artisan operating
the machine. Preferably, the present invention employs
15   TOF-SIMS. Detection of the mass of secondary ions formed
in a TOF-SIMS protocol allows the unique identification
of the corresponding library member, presuming that the
method of construction of the library is known so that an
artisan can assign discrete molecular weights to all
20   molecular species and ionization fragments thereof
(generated in the TOF-SIMS method).

In TOF-SIMS, a pulsed beam of primary ions is
directed to a sample surface. The arriving primary ions
desorb and ionize molecules of the sample present in a
25   monolayer at the surface of the sample. These generated
secondary ions are then accelerated to a uniform energy
by an electric field, and drift through a fixed distance
to a detector. The time-of-flight of these uniform
energy particles through the fixed distance is directly
30   proportional to the charge-to-mass ratio (m/z) of the
ion. Because only the time-of-flight of an ion is
measured to determine its mass, TOF-SIMS provides for
parallel detection of all masses present in a sample, and
an effectively unlimited mass detection range with high
35   mass resolution. Indeed, TOF-SIMS provides a 10^4-10^6 fold
improvement in sensitivity over scanning mass

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spectrometric methods employing other detectors, such as
magnetic sector fields and quadrapoles, which are well
known in the art. TOF-SIMS thus provides a direct mass
spectrometric assay that is generally applicable to

5 reading a wide variety of molecular species assembled in
combinatorial libraries.

     The considerations relevant to use of TOF-SIMS for
such assays are discussed in the literature. For
example, as discussed by Winograd in *Ion Beams and Laser*

10 *Postionization for Molecule-Specific Imaging* (Anal.
Chem., 65, 622A-629A (1993)), an energetic primary ion
bombarding a sample on a solid surface creates a large
amount of damage within 50 Angstroms of the point of
impact. Unless the dose of incident ions is kept below

15 approximately 1% of the number of sample molecules
forming a monolayer, the ion bombardment alters the
surface chemistry. The dose of incident ions of 1% is
referred to as the "static limit." In TOF-SIMS, the
dosage of primary ions remains below the static limit

20 because the incident ion beam is directed toward the
sample as a very short pulse. Use of a pulsed incident
beam is also advantageous because a spectrum with a
dynamic range of several orders of magnitude can be
obtained by the accumulation of a large number of cycles

25 with high repetition rates, as discussed by Benninghoven
et al. in *Surface MS: Probing Real-World Samples* (Anal.
Chem., 65, 630A-639A (1993)). Increased sensitivity may
also be realized using special cationization schemes or
by laser postionization of sputtered neutral molecules,

30 as discussed by Winograd et al., Inst. Phys. Conf. Ser.,
128, 259 (1992).

     The TOF-SIMS technique also allows the primary ion
beam to be focused to a spot size of less than 150 nm,
thereby allowing the concentration of molecules to be

35 mapped over small spatial domains by rastoring the ion
beam across pixels defined on the sample and taking

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spectra at each pixel. Other aspects of TOF-SIMS imaging
are discussed by Chait and Standing in *Time-of-Flight*
Mass Spectrometer for Measurement of Secondary Ion Mass
Spectra (Int. J. Mass Spectrom. Ion Phys., 40, 185-193
5 (1981)); and by Steffens et al. in *A Time-of-Flight Mass*
Spectrometer for Static SIMS Applications (J. Vac. Sci.
Technol., A 3(3), 1322 (1985)).

In certain situations, the information obtained by
TOF-SIMS may not fully distinguish and identify all
10 members of a combinatorial library. For example, various
isomers of a given peptide may be present, each having
the same mass, as, for example, in the case of
phenylalanine-glycine-leucine and glycine-leucine-
phenylalanine. In such situations, TOF-SIMS can be used
15 to determine the sequence of the selected peptide
nonetheless, provided that the library was constructed
from a known set of building blocks. As discussed by
Poppe-Schriemer et al. in *Sequencing an "Unknown" Peptide*
by Time-of-Flight Secondary Ion Mass Spectrometry (Int.
20 J. Mass Spectrom. Ion Phys., 111, 301-315 (1991)), the
parent ions subjected to TOF-SIMS necessarily break down
to the various fragment ions, the masses of which can be
compared and analyzed based on existing mass data to
determine the structure of the selected peptide. This
25 procedure is effective to the extent that the selected
molecular species is one of the possible peptides of the
combinatorial library as determined by the construction
of the library. This procedure is also limited by the
resolving power of TOF-SIMS to distinguish such
30 fragmentions (TOF-SIMS mass accuracy is currently on the
order of ± 0.01 amu, according to Winograd, supra).

Alternatively, an isotope indexing scheme can be
used to differentiate between molecular species that
otherwise have the same mass. For example, to
35 differentiate between phenylalanine-glycine-leucine and
glycine-leucine-phenylalanine, one can either examine the

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fragmentation pattern in the SIMS spectrum or synthesize
one of the peptides using leucine having ^{15}N, an isotope
that is readily distinguished in TOF-SIMS as its atomic
mass is increased by one unit. Distinguishing between a
5   leucine and an isoleucine residue, which are isomers,
necessarily would require such an alternate method.
Similarly, one could use differentially L and D amino
acids, using methods well known in the art.

In particular, the present invention relates to a
10 method of identifying small molecules of a combinatorial
library comprising (a) forming a plurality of complexes
of solid substrates and small molecules, each of which
comprises one substrate, or portion thereof, and one of
said small molecules of said library; and (b) determining
15 the molecular weight of a selected small molecule by
means of secondary ion mass spectrometry. Preferably,
the secondary ion mass spectrometry that is utilized in
the context of the present invention is TOF-SIMS, as
noted above and exemplified below. The small molecules
20 of such a combinatorial library are selected from at
least one of the group consisting of amino acids,
peptides, oligonucleotides, and heterocyclic compounds.
The present inventive method is applicable to small
molecules comprising amino acids that are naturally
25 occurring or synthetic. A preferred combinatorial
library has small molecules that are peptides or
heterocyclic compounds; a more preferred combinatorial
library has small molecules that are peptides.

Suitable peptides comprise as few as two amino acids
30 to as many as about 30; preferably, suitable peptides
comprise from about two amino acids to about fifteen;
most preferably, suitable peptides comprise from about
two amino acids to about ten. Any amino acid may be
incorporated into peptides screened and identified using
35 the present invention, including any combination of the
naturally occurring proteinogenic amino acids as well as

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amino acids not naturally occurring in proteins such as,
but not limited to, dextrorotatory forms of the known
amino acids, for example.

Suitable oligonucleotides consist of as few as two
5   nucleotides to as many as about 50; preferably, suitable
oligonucleotides consist of from about five nucleotides
to about 30; most preferably, suitable oligonucleotides
consist of from about five oligonucleotides to about 15.
Any nucleotide may be incorporated into an
10 oligonucleotide screened and identified using the present
invention, including any combination of the naturally
occurring deoxyribonucleotides and ribonucleotides as
well as those not naturally occurring in biological
systems, such as, but not limited to, H-phosphonate
15 derivatives, N-blocked-5'-O-DMT-deoxynucleoside 3'-(2-
cyanoethyl-N,N-diisopropyl)phosphoramidites, N-blocked-
5'-O-DMT-deoxynucleoside 3'-(2-cyanoethyl-N,N-
diisopropyl)phosphoramidites, N-blocked-5'-O-DMT-
deoxynucleoside 3'-(methyl-N,N-diisopropyl)
20 phosphoramidites, N-blocked-5'-O-DMT-deoxynucleoside 3'-
(2-chlorophenyl) phosphates, N-blocked-5'-O-DMT-
deoxynucleoside 3'-(2-chlorophenyl 2-cyanoethyl)
phosphate, all of which are nucleoside derivatives used
in oligonucleotide synthesis.
25   Suitable heterocyclic compounds consist of, at
minimum, a single four membered ring to as much as a
multiple of four membered or greater membered rings
coupled by carbon chains of 1 to about 20 atoms in
length, such chains being saturated or not. Preferably,
30 suitable heterocyclic compounds include a single four- to
seven-membered ring, as well as, but not limited to
varying combinations of 5, 6, or 7 membered rings having
varying numbers of N, S, or O atoms. More preferably,
suitable heterocyclic compounds include benzodiazepine
35 and derivatives thereof (as, for example, disclosed in
Bunin et al., J. Am. Chem. Soc., 114, 10997-10998

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(1992)), penicillins, cephalosporins, and folate
derivatives. Most preferred, suitable heterocyclic
compounds include benzodiazepine and derivatives thereof,
and angiotensin II receptor antagonists. For example,

5   one angiotensin II receptor antagonist that was developed
to block the renin-angiotensin system for the treatment
of heart failure and possibly chronic renal failure (see,
Weinstock et al., J. Med. Chem., 34, 1514 (1991); Keenan
et al., J. Med. Chem., 36, 1880 (1993)) can be identified

10   in a mixture of other heterocyclic compounds using the
present invention. The formula of the aforementioned
angiotensin II receptor antagonist, ethyl 2-(2'-
thiophenylmethyl)-3-[5'-{(1'-p-carboxyphenylmethyl)-2'-n-
butyl}-imidazolyl]-propenoate, covalently linked to

15   polystyrene beads through various linking moieties is set
forth in Figure 7. The present invention may be applied
to the identification of derivatives of such compunds as
benzodiazepine and the noted angiotensin II receptor
antagonist.

20   Mixed libraries of small molecules comprising amino
acids, peptides, oligonucleotides, and heterocyclic
compounds may be prepared by following standard methods
known to one of ordinary skill in the art. An
oligonucleotide can be, for instance, linked to a peptide

25   through the 5'-hydroxyl of the oligonucleotide. The
peptide end can be modified to include a carboxyl group.
A process of esterification of the carboxyl group with
the 5'-hydroxyl of the oligonucleotide is used to produce
a mixed library containing peptide-oligonucleotide

30   species. Brenner et al., (Proc. Nat'l Acad. Sci. USA,
89, 5381-5383 (1992) also describes a method of
preparation of mixed libraries having nucleotides and
peptides. A mixed library comprising a heterocyclic
compound and a peptide is also prepared by the reaction

35   of suitable functional groups present on the heterocyclic
compound. For instance, the carboxyl group on a

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heterocyclic compound is reacted with the amino group on the peptide to provide an amide linkage.

The small molecules of the combinatorial library preferably are linked covalently to the substrate, using

5   methods well known in the art. A preferred covalent linkage between the small molecule and the substrate has the characteristic of being able to break in response to external changes at levels that do not modify substantially the structure of the small molecules of the

10 combinatorial library. Such a covalent linkage may be effected, for example, by means of a suitable linking moiety that couples both to the small molecule and the substrate or a substrate having suitable reactive groups coupled thereto. In essence, a suitable covalent linkage

15 will break conditionally. When a linking moiety or a substrate-bound reactive group is used, the covalent bonds between the small molecule and the substrate will break at one or more of its internal covalent bonds or a bond that it forms with either the substrate or the small

20 molecule or both, thereby destroying any covalent linkage between the small molecule and the substrate. At least an appreciable proportion of the population of small molecules will be fully free of the covalent linkage, however, some or even a majority of the small molecules

25 may remain attached covalently. The proportion of small molecules whose covalent linkage to the substrate are broken, however, may remain associated with the substrate by weaker molecular interactions, such as, but not limited to physical adsorption, hydrophobic interactions,

30 and van der Waals forces. Suitable condition changes that may be used to effect the bond break or breaks of the covalent linkage include effective levels of temperature, electromagnetic radiation, sound or acidity at a level that leaves the small molecules of the library

35 intact but still in association with the substrate via

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some combination of the aforementioned or other weak
molecular interactions.

Suitable linking moieties are those that comprise a
reactive functional group selected from the group
5   consisting of alcohol, amino, carboxyl, acetal,
thioacetal, and aminoalkyl, aralkyl, amino aralkyl, and
haloalkyl, and a nitroaromatic group having a benzylic
hydrogen ortho to the nitro group, such as o-nitrobenzyl
derivatives, and benzylsulfonyl derivatives; and are
10 cleavable by suitable vapor or photochemical means.
Preferably, the linking moiety comprises at least one
reactive group that is selected from the group consisting
of hydroxyl, amino, carboxyl, acetal, thioacetal, C_1-C_{10}
alkylamino, C_1-C_{10} aralkylamino, and C_1-C_{10} haloalkyl, and
15 an ortho-nitrobenzylic group having a benzylic hydrogen.
Photoremovable groups are discussed in U.S. patent
5,143,854 to Pirrung et al., for example.

In particular, suitable linking moieties include p-
alkoxybenzyl alcohol (used in the Wang resin), F-moc-2,4-
20 dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine, F-moc-4-
methoxy-4'-(gamma-carboxypropyloxy)-benzhydrylamine, 4-
hydroxymethyl-phenoxy-acetic acid, aminomethyl (used in
the PAM resin), benzhydrylamine, Cl-CH₂-Ph-(used in
Merrifield resin), benzylacetal (used in the Acetal
25 resin), benzylthioacetal (used in the Thioacetal resin),
and 2-methoxy-4-alkoxybenzyl alcohol (used in Sasrin®
resin). Preferred linking moieties include F-moc-2,4-
dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine, F-moc-
methoxy-4'(gamma-carboxypropyloxy)benzhydrylamine, p-
30 alkoxybenzyl alcohol, benzhydrylamine, Cl-CH₂-Ph, 2-
methoxy-4-alkoxy benzyl alcohol, 6-nitroveratryloxy
carbonyl, 2-nitrobenzyloxycarbonyl, and α,α-dimethyl-
dimethoxybenzyloxycarbonyl, more preferred linking
moieties include 2-methoxy-4-alkoxybenzyl alcohol. It is
35 appreciated that different linker chemistry may enhance
the molecular ion signal of covalently attached species.

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The covalent linkage between the substrate and the
small molecule may also be mediated by the reactive group
or groups attached to the substrate. For example, as
recited above, the polystyrene-derivative bead known as

5   Sasrin® (Bachem Biosciences) has a reactive group
(2- methoxy-4-alkoxy benzyl alcohol) that covalently
couples to carboxylic acid groups found on all peptides.
The covalent bond formed by the coupling of these two
groups is acid labile. Accordingly, the exposure of TFA

10   vapor to a small molecule covalently bound to a Sasrin®
bead results in the breaking of certain covalent bonds
associated with the Sasrin® bead, i.e., the linking
moiety, thereby releasing an intact molecular species.
TFA is used preferably as a dilute solution in a

15   suitable organic solvent. The concentration of TFA is
preferably kept in the range of about 0.5% to about 2% by
weight, and more preferably from about 0.75% to about
1.5%, and most preferably from about 0.9% to about 1.1%
by weight of the solution. The TFA is applied in concert

20   with a means for swelling the polystyrene beads, such as,
for example, dissolving the TFA in an organic solvent.
Preferred organic solvents include halogenated lower
aliphatic hydrocarbons having 1-3 carbon atoms, including
methylene chloride, chloroform, dichloroethane, 1,1,2,2-

25   tetrachloroethane, trichloroethylene,
tetrachloroethylene, and the like, with methylene
chloride being more preferred.
The substrate upon or with which the small molecules
of the combinatorial library are synthesized and/or

30   associated may be any suitable substrate, including, but
not limited to, resin, polystyrene, Sasrin®, Wang resin,
Pam resin, and Merrifield resin, further including
suitable combinations thereof. Such resins are
commercially available from Bachem Bioscience Inc., for

35   example. The substrate used in the present invention may
be formed into any suitable shape, including, but not

20

limited to, spheres, cubes, rectangular prisms, pyramids,
cones, ovoids, sheets, and cylinders. Particularly when
the substrate is used in the form of a sheet, such as
when placed on the surface of a glass microscope slide,

5   defined portions of the sheet may be apportioned for
different small molecules of a combinatorial library, as
disclosed in Fodor et al., supra. Preferably, the
substrate as used in the present invention is formed into
small particles that occupy no more than nine ten

10 thousandths of a cubic millimeter, such as a sphere
having a diameter of 120 microns, each of which has
associated thereto a single small molecule structure.
More preferred, the substrate used in the present
invention is a bead or sphere having a diameter that is

15 from about 10 microns to about 120 microns. Most
preferred, the substrate used in the present invention is
a bead or sphere having a diameter that is from about 20
microns to about 80 microns.

        The present invention also relates to the linking
20 moiety per se. The characteristics and examples of the
linking moiety are the same as discussed above relative
to the method for identifying individual small molecules.

        The following examples further illustrate the
present invention and, of course, should not be construed
25 in any way as limiting its scope.

                            Example 1

        This example illustrates the use of TOF-SIMS for the
identification of·the molecular weights of combinatorial
30 library constituent molecules bound to the polystyrene
bead surfaces. TOF-SIMS is an instrument that is well
known in the art and available from various commercial
sources. Accordingly, an artisan may use any such TOF-
SIMS in accordance with the specific machine's operating
35 instructions. What follows is a description of the use
of one TOF-SIMS instrument manufactured by Kratos, Inc.

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(Ramsey, NJ), which was used in the context of the
present invention.

A schematic diagram of the apparatus is shown in
Figure 1. An ion gun 100 is used to generate a beam of
5   primary ions directed at a bead 101 coated with a
monolayer of the sample. The ion gun 100 is illustrative
of the liquid metal type (LMIG), and provides a source of
Ga^+ ions having an energy of 25 keV. The dosage of these
ions is limited to stay within the static limit by
10 limiting sample exposure to 200,000 pulses of 500 pA
current and 20 ns duration per pulse. This exposure
corresponds to 10^7 Ga^+ ions focused into a circular area
of 40 μm diameter (the diameter of the bead) or 8 x 10^{11}
Ga^+ ions/cm^2. A 20 ns primary ion pulse yields a mass
15 resolution of ~1500 at m/z 100. Pulsing of the beam is
achieved by rapid electrical deflection of the beam
through an aperture for the desired pulse duration. The
ion beam is focused to a spot size of approximately 150
nm on the surface of the bead 101 through focusing optics
20 denoted generally by reference numeral 102. Since a
plurality of beads are held on a single copper grid, the
ion gun beam may be rastored across the surface, with
spectra being taken at each pixel to determine the
surface constituents there.
25   Bombardment of a bead 101 by the ion beam causes the
liberation of secondary ions from the surface. Secondary
ions liberated from the surface of the bead 101 by the
incident ion pulse are then accelerated to a uniform
energy and are focused by an extraction lens 105. This
30 lens is a combination of a flat extraction plate and an
enzel lens. As will be discussed in greater detail
below, a constant voltage is maintained between the
copper grid 106 to which the bead is attached and the
extraction lens 105. Preferably, the distance from the
35 grid 106 to the extraction lens 105 is about 3mm. Once
through the extraction lens 105, the uniform nergy ions

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travel along the linear path designated generally by
reference numeral 110. Focusing optics, preferably in
the form of a reflectron 120, are placed at the end of
the path 110. These focusing optics correct for angular
5   distribution of the secondary ions, as described in
Cotter, *Biomed. Environ. Mass Spec.*, 18, 513-532 (1989).
The focused secondary ions are then detected by a
channelplate detector 130 located at the end of the TOF
analyzer defined by path 110 and reflectron 120.
10   Preferably, the length of the TOF analyzer is about 2m.
The channelplate detector 130 is connected to a computer
140, which performs processing required for spectrum
analysis. Further electronics, not shown, are used for
synchronizing the system so that the time between
15   generation of secondary ions and their arrival at the
channelplate detector 130 is accurately measured.

To accelerate the secondary ions to a uniform
energy, a constant voltage is maintained between the
substrate 106 and the extraction lens 105. This voltage
20   is preferably 7200 volts, with the copper grid being held
at +2.5 kV, and the extraction lens being held at -4.7kV
(for positively charged secondary ions). The polarity
and magnitude of these signals may be changed to allow
for detection of negatively charged species. Two
25   mechanisms present in this configuration can lead to
higher signals at the leading edge of a given bead.
Because the ion current densities generated by the ion
gun 100 are quite large, some charging of the sample
occurs during bombardment. Further, because the bead has
30   a physical dimension (illustratively a 40 micron
diameter) in the 3mm extraction gap, a voltage gradient
on the order of 150 V across the bead may be present.
The size of this gradient is affected by the size and
shape of the bead and the angle of incidence of the Ga^+
35   ion. To compensate for charging of the bead, the sample
may be flooded periodically with low energy electrons,

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such as 50 nA/cm^2 of 30 eV electrons for 50 μs between
each Ga$^+$ ion pulse, to eliminate charging artifacts.

Example 2

5   This example illustrates the TOF-SIMS spectrum of a
40 micron polystyrene bead coated with an approximately
one molecular layer of phenylalanine.

Standard 40 micron diameter polystyrene beads
(Bachem Bioscience) were treated with a solution of
10   phenylalanine to cover the beads with a monolayer of the
amino acid by physical adsorption, as follows:
Polystyrene beads were immersed in a 10^{-4} M methanol
solution of phenylalanine, removed after several minutes,
allowed to air dry, and then placed on a copper grid for
15   analysis. For these measurements, the dose of incident
25 keV Ga$^+$ ions was controlled by limiting sample
exposure to 200,000 pulses of 500 pA current and 20 ns
duration per pulse. This exposure corresponds to 10^7 Ga$^+$
ions focused into a circular area of 40 μm diameter or 8
20   x 10^{11} Ga$^+$ ions/cm^2. A 20 ns primary ion pulse yields a
mass resolution of ~1500 at m/z 100. The low dose of
primary ions ensures that sample damage does not alter
the chemical nature of the target surface, as noted by
Benninghoven and Sichterman (Anal. Chem., 50, 1180
25   (1978)).

As shown in Figure 2A, the resultant TOF-SIMS
spectrum exhibits large peaks at m/z 120 (M-CO$_2$H)$^+$, 166
(M+H)$^+$, 188 (M+Na)$^+$, and 210 (M+H+Na$_2$)$^+$. Other peaks
characteristic of bulk polystyrene (labeled "PS" at m/z
30   91 (C$_7$H$_7$), 103, 105, 115, 117, 127, 128, 129, 141, 152,
165, 178, 190, and 193; see Leggett et al., J. Chem. Soc.
Faraday Trans., 88, 297 (1992)), sodium (at m/z 23),
potassium (at m/z 39), and copper (at m/z 63 and 65) are
also assignable.

35   Although sensitivity of the TOF-SIMS technique
varies depending on the molecular character of the sample

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being tested, it is noteworthy that for phenylalanine
adsorbed on a polystyrene bead, the detection limit was
approximately 500 attomoles on the bead surface.
Considering the capability of a 40 micron sphere to have
5   adsorbed to it at least 50 femtomoles, i.e., at least 100
times above the detection limit, the TOF-SIMS technique
was shown hereby to have the requisite sensitivity for
analyzing combinatorial libraries according to the
present invention.

10   Thus, this example illustrates the capability of
TOF-SIMS to analyze small quantities of amino acids
adsorbed on beads.

                           Example 3

15   This example illustrates the TOF-SIMS spectrum of a
40 micron polystyrene bead coated with an approximately
one molecular layer of the tripeptide, valine-tyrosine-
valine (V-Y-V).

          Standard 40 micron diameter polystyrene beads
20   (Bachem Bioscience Inc.) were treated with a solution of
V-Y-V to cover the beads with a monolayer of the
tripeptide by physisorption and then placed on a copper
grid, as described in Example 2. For the TOF-SIMS assay,
the pulsed Ga^+ ion beam was rastored across the 100
25   micron field, during which time a TOF-SIMS spectrum was
recorded (Figure 3) for each ~1 square micron pixel. An
image was rendered by mapping the intensity of (M+H),
$(M+Na)^+$, and $(M+H+Na_2)^+$ ions at m/z 380, 402 and 424,
respectively. For V-Y-V, the intensity was generally 0-4
30   counts per pixel. In spite of these relatively low count
rates, a clear image of the coated bead was discerned
easily in a photograph of the noted intensity levels
recorded and digitized in each pixel.

          A number of points important in the interpretation
35   of results derived from the inventive method can be made
with reference to the V-Y-V analysis. First, although

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the copper grid is electrically conductive, the
polystyrene bead itself is an electrical insulator
subject to charging. Normally, in static TOF-SIMS
experiments, charging is not a significant problem, due
5   to the small number of incident ions needed to record a
spectrum. For imaging of small areas, as done to
generate the image just mentioned, however, ion current
densities are much higher, therefore some type of charge
compensation is essential. In the experiments
10 accomplished in the course of elucidating the present
invention, the sample was flooded with 50 nA/cm^2 of 30 eV
electrons for 50 microseconds between each Ga$^+$ ion pulse
to eliminate charging artifacts, after the methods
disclosed in Gardella and Hercules (Anal. Chem., 52, 226
15 (1980)) and Briggs and Wooton (Surf. and Int. Anal., 4,
109 (1982)).

Second, the influence of the shape of the particle
on which the molecular species of the library are
attached and the angle of incidence of the Ga$^+$ ion stream
20 have an impact on results. In the configuration used in
the context of the present inventive method, the Ga$^+$ beam
was incident at 45° from the surface normal to generate
the data displayed in Figure 3. For example, a
polystyrene sphere of ~60 microns in diameter, placed in
25 a 3mm extraction gap, will have a field of 7200 volts
applied across it. Accordingly, in addition to problems
dictated by the morphology of the bead, there is a 150
volt field gradient across the bead. Both of these
effects tend to produce higher signals near the leading
30 edge of the bead, as is visualized in the digitized
images shown in Figure 4, for example.

Third, each of the TOF-SIMS assays reported herein
was completed in less than 4 minutes. The analysis time
is determined by the flux of incident ions and the time
35 required to reach the damage threshold. For small beads

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and/or higher current sources, the analysis time could be
reduced significantly by about an order of magnitude.

        Similar results to those shown in Figure 3 have been
obtained using glycine-proline-glycine-glycine, as well
5   as a variety of other small peptides. The technique for
larger peptides, such as bradykinin, for example, having
11 amino acid residues, provided a recognizable TOF-SIMS
spectrum when the 11-mer was adsorbed onto a polystyrene
film (see Steffens et al., supra). Because combinatorial
10 libraries of peptides on polystyrene beads generally
consist of linear chains of only three to six amino
acids, the range imaged by TOF-SIMS is certainly
sufficient to determine the parent molecular ion of the
adsorbed peptides of such libraries.

15   Accordingly, using the tripeptide V-Y-V, this
example provides elucidation of important parameters in
the direct imaging of a combinatorial library of peptides
adsorbed onto polystyrene beads. One must be cognizant
of the charging capacity of the substrate to which the
20 molecular species of a library are adsorbed because of
the substrates' capacity to increase the ion current
density. Additionally, the shape of the substrate used
and the angle of incidence of the Ga^+ ion can tend to
produce artificially higher signals, and therefore must
25 be compensated for using methods well known in the art.
Finally, the time per TOF-SIMS was only four minutes, and
could be reduced significantly, which is one of the
surprising improvements that the present invention
provides to the field of combinatorial library screening
30 and analysis.

                          Example 4

        This example illustrates the determination of the
molecular weight of a peptide at a particular address,
35 using the TOF-SIMS assay as described in Example 2 and a

novel method to reversibly yet covalently link small
molecules of a combinatorial library to a substrate.

Combinatorial libraries constructed on polyester
beads are necessarily bound covalently thereto at least
5   during the construction reactions. For the determination
of the molecular weight of a small molecule of such a
library located at a particular address, i.e., at a ·
particular site on a grid, it is necessary to break the
covalent linkage in order to desorb intact molecules.
10 For the purpose of testing requirements of an address-
based determination, phenylalanine was adsorbed onto
Sasrin® polystyrene beads and linked covalently thereon
by means of reactive groups attached to the Sasrin®
beads, using the methods of Mergler et al. I (Tet. Lett.,
15 29, 4005 (1988)) and Mergler et al. II (Tet. Lett., 29,
4009 (1988)).

The formation of the covalent bond between
phenylalanine and the Sasrin® bead dramatically reduced
the yield of molecular ions in the SIMS spectrum. This
20 effect is shown in Figure 2B where the yield of $(M+H)^+$ at
m/z 166 is no longer visible, although strong fragment
ions are found at m/z 120. Accordingly, the parent
compound, phenylalanine, could not be identified. When
larger molecules were tested in otherwise identical
25 experiments, parental molecular ions were not observed
and the spectra were found to consist mainly of intense
fragment ions from protecting groups and monomers, such
as amino acids. For example, with the pentapeptide,
leucine-serine-arginine-isoleucine-valine, the expected
30 parental molecular ion at 587 m/z was not observed nor
were any of the cationized species, although several
fragment ions typical of each of the monomer units were
found in the low mass range. See Mantus et al., Anal.
Chem., 65, 1431 (1993). Hence, the TOF-SIMS analysis of
35 small molecules covalently bound to polyester beads was

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determined to be ineffective unless the covalent bond is broken prior to the TOF-SIMS analysis.

A protocol for clipping the covalent bond or bonds that bind a small molecule of a library to a suitable

5   substrate, while leaving the small molecule resting in place on the substrate, was developed using phenylalanine attached to a Sasrin® polystyrene bead to test the protocol. Beads with covalently attached amino acids were then transferred to a copper grid. The copper grid

10 was used as a support and markings on the grid were used to locate specific beads, i.e., addresses.

It was discovered that Sasrin® polystyrene beads form acid sensitive covalent bonds with peptides, for example. The beads having small molecules covalently

15 attached thereto were placed in a chamber saturated with trifluoracetic acid (TFA) and methylene chloride (CH_2Cl_2) vapors from a 2% TFA in CH_2Cl_2 solution. A three minute exposure was sufficient to cleave the amino acid from the bead. The progress of the reaction was monitored by

20 observing a color change from off-white to purple on the beads themselves. Once the cleavage reaction was complete, the beads and copper grid were inserted directly into the TOF-SIMS instrument for analysis.

The mass spectra of the beads subjected to the vapor

25 phase clipping exhibited a strong signal for each corresponding parent ion. The SIMS spectrum of the clipped phenylalanine is shown in Figure 2C, while the corresponding image of m/z 166 is shown in Figure 4. An important observation derived from Figures 4A and 4B,

30 which are TOF-SIMS images directed at the identical address, but using different filters, is that the peptide is confined to the bead. This is evident because its signal (shown in Figure 4A) is not found from the surrounding copper grid (shown in Figure 4B). Therefore,

35 the phenylalanine after breaking its covalent linkage to the polyester bead remained associated with the bead, due

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to physical adsorption or other weak molecular effects.
Moreover, the signals observed for phenylalanine at m/z -
- (M+H)$^+$ in Figure 2C are more intense than the same
signal for phenylalanine when compared to the signals

5   when it was simply physically adsorbed to a bead (Figure
2A), perhaps due to the better uniformity of coverage on
the bead resulting from the covalent bond formation.
Therefore, greater sensitivity results from analyzing
molecular species clipped from the beads than from

10   analyzing those prepared by other methods, such as by
physisorption alone.

The technique was further tested by imaging a
mixture of phenylalanine and leucine coated beads, using
the same procedure as above. The beads were placed on a

15   copper grid and cleaved with TFA as described above. The
image is shown in Figure 5, which is a field of view of
120 microns. Even though the beads were very close to
each other there was no significant cross contamination,
as seen by comparing Figure 5A with Figure 5B, wherein

20   the (M+H)$^+$ ion intensity for leucine is shown in Figure
5A and the (M+H)$^+$ ion intensity for phenylalanine is
shown in Figure 5B. As in Figure 4, the images depicted
in Figure 5 are at the identical address, using different
filters.

25   This example illustrates a method for further
increasing the sensitivity of the TOF-SIMS molecular
weight assay and, more significantly, illustrating a
method for the determination of the molecular species
contained in a library found at particular locations on a

30   grid of beads containing different molecular species.

30

Example 5

This example illustrates the TOF-SIMS assay as
applied to the identification of a tripeptide covalently
bound to a bead.

5   The tripeptide Val-Tyr-Val was covalently attached
through an acid sensitive linker to the bead, according
to the method described in Example 4. The bead was
subjected to clipping by the vapor phase method and
subjected to characterization by TOF-SIMS, as described

10   in Example 2. The mass spectrum displayed in Figure 6
(lower panel) shows ions at m/z 380 (M+H), 281, and 263.
The assignment of these peaks is shown in the figure,
which are the whole tripeptide, a Val-Tyr dipeptide
fragment, and a Tyr-Val dipeptide fragment, respectively.

15   In the low mass range (Figure 6, upper panel), intense
peaks were found at m/z 72 (Val-Co_2H) and 136 (Tyr-Co_2-H).
Using the method of Biemann et al., Mass Spectrom. Rev.,
6, 1 (1987), analysis of the TOF-SIMS spectrum of
fragment sequence ions provided not only the composition,

20   but the Val-Tyr-Val sequence through the above
fragmentation pattern.

Accordingly, this method provided a determination of
the mass of the parent ion and thereby demonstrates a
method to identify directly those members of a library

25   with a given molecular weight, as illustrated herein.

Example 6

This example illustrates the use of the electrospray
mass spectrometry to the identification of a heterocyclic

30   small molecule covalently bound to the Sasrin® bead for
comparison to the instant invention. The data disclosed
in this example and Example 7 were provided by S.A. Carr,
M.E. Hemling, G.D. Roberts, and J. Weinstock of the
Chemical and Biological Research Division of SmithKline

35   Beecham Pharmaceuticals, King of Prussia, Pennsylvania.

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A bead having attached thereto angiotensin II
receptor antagonist (ethyl 2-(2'-thiophenylmethyl)-3-[5'-
{(1'-p-carboxyphenylmethyl)-2'-n-butyl}-imidazolyl]-
propenoate) was isolated and transferred to a micro-
5   Eppendorf tube. The analyte was permitted to cleave for
15 min with 1% TFA in methylene chloride. The sample was
dried, and the compound was extracted/dissolved in 10 μl
acetonitrile. One-tenth of the solution was introduced
by flow injection and analyzed by ESMS on a Perkin Elmer
10   Sciex API-III triple quadrupole analyzer (Thornhill,
Ontario). An intense signal corresponding to the (M+H)+
ion was readily detected at m/z 453.18 (theor. 453.18),
as set forth in Figure 8A. Strong signals from the bead
and the linker were also observed in the range m/z 500 to
15   600. An additional 10% of the solution was then analyzed
by tandem MS on the same quadrupole instrument and the
result is set forth in Figure 8B. The molecular ion
cluster was selected by Q1 of the triple quadrupole and
collisionally activated with the argon in the collision
20   cell, Q2. The product ions were detected in Q3. A large
number of fragment ions were observed, all of which were
readily assigned to the structure of the angiotensin II
receptor antagonist.

Thus, electrospray mass spectrometry could be used
25   to determine the molecular weight of covalently bound
heterocyclic compound.

Example 7
This example illustrates the identification of the
30   angiotensin II receptor antagonist covalently bound to
the Sasrin® bead by using the MALDI method, presented
here for comparative purposes.

The Sasrin® bead was placed on a stainless steel
sample target and exposed for about 1 hr to TFA vapor in
35   an enclosed chamber. A 0.5 μl alignment of a solution
of dihydroxybenzoic (DHB) acid matrix in acetone was

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placed on the bead, and allowed to dry in air. Analyses
were carried out using two types of Fisons VG MALDI mass
spectrometers (Manchester, UK), both single-stage
reflectron instruments using photon irradiation from a
5   337-nm pulsed nitrogen laser and 23-keV accelerating
voltage. For generating conventional MALDI spectra, a
low performance TOFSpec with maximum mass resolution in
the reflecting mode of M/Δn 1200 (FWHM) was used.   A
conventional MALDI mass spectrum was generated by 41
10   laser shots that were averaged to produce the spectrum
set forth in Figure 9A. The instrument was mass
calibrated externally using the (M+H)+ peaks of DHB and
gramicidin S. A significant signal is observed for the
(M+H)+ at m/z 453.15 (theor. 453.18). Fragments were not
15   detected. Peaks below m/z 200 are primarily due to the
DHB matrix. DHB has been found to be particularly useful
for low M_r organic compounds because it gives a very low
matrix background above m/z 200.

     To overcome the fact that MALDI did not produce a
20   significant fragment spectrum for this compound, the post
source decay (PSD) method (see e.g., Della-Negra et al.,
Anal. Chem., 57, 2035 (1985); Tang et al., Anal. Chem.,
60, 1791 (1988); Spengler et al., J. Phys. Chem., 96,
9678 (1992); Kaufmann et al., J. Mass Spectrom., 131, 355
25   (1994)) was applied to the angiotensin II receptor
antagonist analysis using a VG TofSpec-SE instrument. An
approximately 10 Da window centered on the parent ion at
m/z 453.2 was selected for product ion analysis using a
Bradbury-Nielson ion gate. PSD mass spectra were
30   acquired in seven consecutive, overlapping mass scale
segments, each representing a ca. 30% mass change from
the previous segment. The PSD segments were combined and
externally mass calibrated against a PSD spectrum of
renin substrate tetradecapeptide by the data system to
35   yield Figure 9B. The spectrum was readily interpreted

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using well-developed rules for the interpretation of
fragment ions.

Thus Example 7 illustrates that the covalently bound
heterocyclic compound can be clipped by TFA and its
5   molecular weight determined by MALDI.

Example 8

This example illustrates the identification of the
angiotensin II receptor antagonist bound to the Sasrin
10 bead by the TOF-SIMS method.

TOF-SIMS studies of the beads were carried out after
exposure to the beads of TFA vapor for a period of 2
hours in an enclosed chamber. The beads were supported
on a small piece of silicon wafer. After the TFA vapor
15 treatment, the wafer was transferred directly into the
SIMS analysis chamber of a Kratos Prism TOF-SIMS
instrument, and the analysis was carried out. A pulsed
25-keV primary ion beam (minimum beam diameter 200 nm)
irradiated the sample; the pulse width was 7 ns. A 2.5-
20 keV stage voltage was used to accelerate the ions into
the analyzer, which is a reflectron device capable of
mass resolution better than $m/\Delta m = 10,000$. The
analytical data were output as either mass spectra or
mass-resolved images.

25   Figure 10 sets forth the positive ion spectrum
obtained from a single bead with a diameter of about 50
μm that had been obtained by bombarding with 2.2×10^7 Ga^+
ions. Assuming 10^{14} molecules cm^{-2}, less than 0.5% of the
surface has experienced an ion impact. This is well
30 within the static limit for SIMS. The (M+H)+ at m/z 453
is clearly evident together with the significant fragment
peaks at m/z 283, 135 and 97. The internal calibration
procedure using the H+ and the CH_3+ ions yield a m/z for
the (M+H)+ ion of 453.18 (theor. 453.18). The accuracy
35 of the calibration was checked by incorporating a CsI
internal standard and using the Cs+ at m/z 133. The

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fragment masses in Figure 10 can be determined with
similar accuracy, which also aids both in their
assignment and in their use along with the molecular mass
in defining the identity of the target compound.

5   The calculated and observed masses of fragment ions
of the angiotensin II receptor antagonist detected in the
illustrative examples 6-8 are set forth in Table 1 below.
It is clear that the errors in the masses are negligible
and that the three mass spectrometric techniques

10   described above are suitable for identifying polymer
bound small molecules that may be present at picomole
quantities. The TOF-SIMS method has even greater
sensitivity than MALDI as shown by the smaller magnitude
of the error in the experimental masses.

15

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Table 1. Calculated and observed masses of fragment ions in Figures 8B, 9, and 10A

Composition	Calc. Mass	Experimental masses (errors)		
		ESMS[b]	MALDI[b]	TOF-SIMS
C_5H_5S	97.0112	96.96 (0.05)	97.0 (0.0)	97.015 (0.004)
$C_8H_7O_2$	135.0446	134.99 (0.05)	135.1 (0.1)	135.045 (0.000)
$C_{11}H_{15}N_2O_2$	207.1133	207.02 (0.09)	207.1 (0.0)	not obsd.
$C_{11}H_{19}N_2O_2$	235.1447	235.07 (0.07)	235.2 (0.1)	not obsd.
$C_{15}H_{16}N_2O_1S$	272.0983	272.06 (0.04)	272.1 (0.0)	not obsd.
$C_{17}H_{19}N_2O_2$	283.1446	not obsd.	not obsd.	283.144 (0.001)
$C_{19}H_{21}N_2O_4$	341.1501	341.17 (0.02)	v. weak	not obsd.
$C_{21}H_{25}N_2O_4$	369.1814	369.22 (0.04)	weak	not obsd.
$C_{23}H_{23}N_2O_3S$	407.1429	407.21 (0.07)	407.1 (0.1)	not obsd.
$C_{23}H_{25}N_2O_4S$	425.1535	425.22 (0.07)	425.0 (not measured)	not obsd.
$C_{25}H_{29}N_2O_4S$	453.1848	453.18 (0.00)[a]	453.15 (0.03)[a]	453.183 (0.002)

[a] Values were obtained from normal spectra, not MS/MS or PSD spectrum.
[b] ESMS and MALDI data were provided by S.A. Carr, M.E. Hemling, G.D. Roberts, and J. Weinstock of the Chemical and Biological Research Division of SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania.

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Figure 11 sets forth two images obtained by
collecting secondary ions as the primary beam is rastored
across the bead surface. The images show the ion
collection distribution for (A) the (M+H)+ ion and (B)
5   the m/z 135 ion. It is clear that the ions attributable
to the target compound are collected primarily from the
bead and few ions are evident from the silicon support.
Thus, the images demonstrate that even after the TFA
treatment, the target compound remains substantially on
10   the bead.

Thus, Example 8 illustrates that a heterocyclic
compound covalently bound to a substrate can be clipped
by TFA and its molecular weight determined by TOF-SIMS.

15   Example 9

This example sets forth the results of a TOF-SIMS
assay of the present invention directed to the
aforementioned angiotensin II receptor antagonist
covalently attached to the Wang resin, the Acetal resin,
20   or the Thioacetal resin.

Angiotensin II receptor antagonist-substrate
constructs were constructed using the Wang resin, the
Acetal resin, or the Thioacetal resin as substrate. The
antagonist-substrate covalent linkage was effected using
25   linkers associated with the respective linkers, as shown
in Figure 7. The covalent linkages were clipped by
exposure to TFA vapor. TOF-SIMS was applied to each of
the above resin samples and the molecular weight of the
antagonist was determined successfully.
30

The contents of each of the references identified
herein are hereby incorporated by reference in their entirety.

The invention being thus described, it will be
obvious that the same may be varied in many ways. Such
35   variations are not to be regarded as a departur from the
spirit and scope of the invention, and all such

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modifications as would be obvious to one skilled in the
art are intended to be included within the scope of the
following claims.

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WHAT IS CLAIMED IS:

1.   A method of identifying individual small molecules of a combinatorial library comprising:

(a)   forming a plurality of complexes of solid
5   substrates and said small molecules, each of said complexes comprising one substrate, or portion thereof, and at least one of said small molecules of said combinatorial library, wherein said substrate and said small molecule are attached to one another by a covalent
10   or ionic bond;

(b)   breaking said covalent or ionic bond such that said small molecule remains physically adsorbed to said substrate; and

(c)   determining the molecular weight of a
15   selected small molecule that remains physically adsorbed to said substrate by means of a secondary ion mass spectrometry.

2.   The method of claim 1, wherein said small
20   molecules are selected from the group consisting of amino acids, peptides, oligonucleotides, heterocyclic compounds, and combinations thereof.

3.   The method of claim 2, wherein said small
25   molecules are covalently bonded to said substrates.

4.   The method of claim 3, wherein said substrate comprises a polymeric resin having a linking moiety attached thereto.

30

5.   The method of claim 4, wherein said polymeric resin is a polystyrene resin having a linking moiety attached thereto.

35   6.   The method of claim 5, wherein said linking moiety comprises at least one reactive group that is

selected from the group consisting of hydroxyl, amino,
carboxyl, acetal, thioacetal, C_1-C_{10} alkylamino, C_1-C_{10}
aralkylamino, and C_1-C_{10} haloalkyl, and an o-nitrobenzylic
group having a benzylic hydrogen.

5

7.   The method of claim 6, wherein said linking
moiety is selected from the group consisting of F-moc-
2,4-dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine, F-
moc-methoxy-4'(gamma-carboxypropyloxy)benzhydrylamine, p-

10   alkoxybenzyl alcohol, benzylacetal, benzylthioacetal,
benzhydrylamine, Cl-CH_2-Ph, 2-methoxy-4-alkoxy benzyl
alcohol, and o-nitrobenzyloxy carbonyl.

8.   The method of claim 7, wherein said linking

15   moiety is selected from the group consisting of 2-
methoxy-4-alkoxy benzyl alcohol, benzylacetal, and
benzylthioacetal.

9.   The method of claim 8, wherein said covalent

20   bond is broken without substantial modification of said
small molecule.

10.   The method of claim 9, wherein said covalent
bond is broken by using a vapor comprising trifluoracetic

25   acid.

11.   The method of claim 10, wherein said covalent
bond is broken by using a mixture of trifluoracetic acid
and methylene chloride vapors.

30

12.   The method of claim 11, wherein said substrate
is a bead.

13.   The method of claim 12, wherein said bead has a

35   diameter of from about 10 microns to about 120 microns.

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14.   The method of claim 12, wherein said secondary
ion mass spectrometry is time-of-flight secondary ion
mass spectrometry.

5   15.   The method of claim 14, wherein said method
further comprises mapping of the spatial distribution of
said small molecules on said beads.

16.   The method of claim 13, wherein said small
10   molecule is an amino acid or a peptide.

17.   The method of claim 16, wherein said peptide
comprises two to ten amino acids.

15   18.   The method of claim 17, wherein said method
further comprises determination of the sequence of said
peptide from the fragmentation pattern obtained in said
time-of-flight secondary ion mass spectrometry.

20   19.   The method of claim 14, wherein said small
molecule is a heterocyclic compound comprising four to
seven membered rings having N, S, or O, and combinations
thereof.

25   20.   The method of claim 1, wherein said substrate
is a polystyrene bead having a reactive group, said small
molecule is an amino acid, peptide, oligonucleotide, or a
heterocyclic compound, or a combination thereof, said
covalent bond is an acid sensitive ester bond, said
30   covalent bond is broken by exposing said complex placed
on a grid to the vapors of trifluoracetic acid and
methylene chloride, and said secondary ion mass
spectrometry is time-of-flight secondary ion mass
spectrometry.

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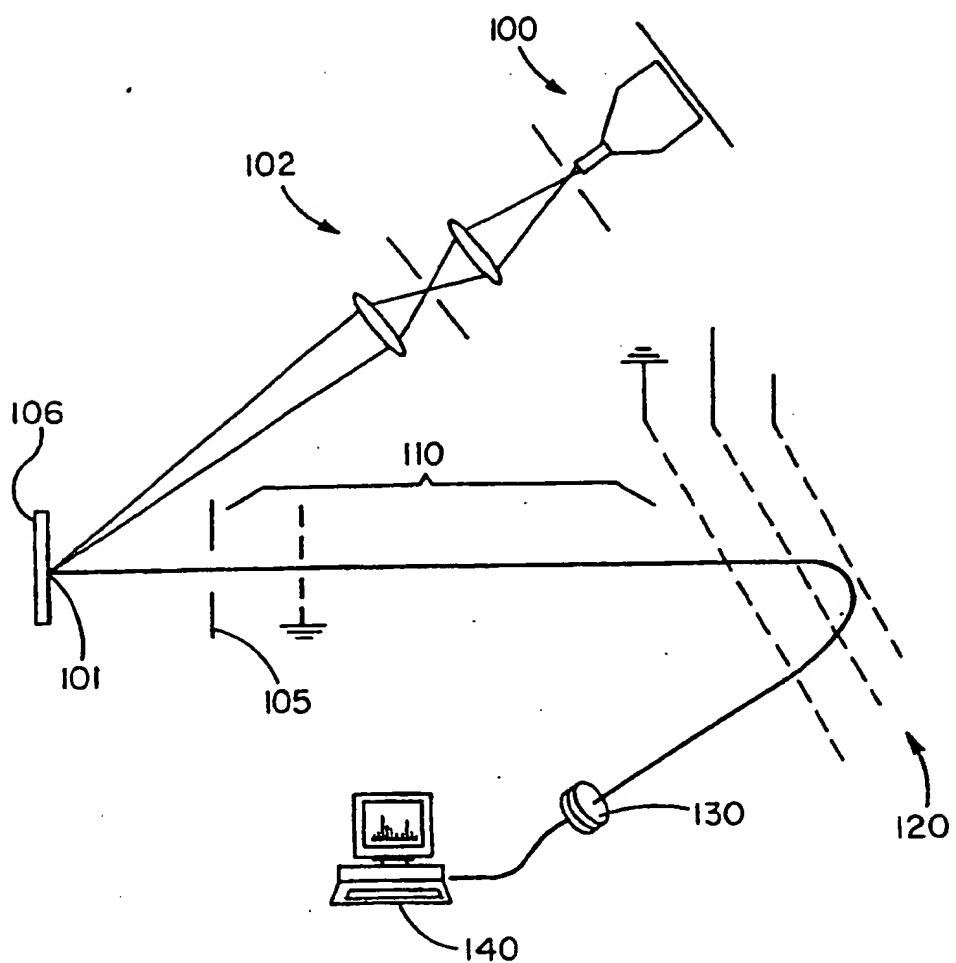
FIG. 1

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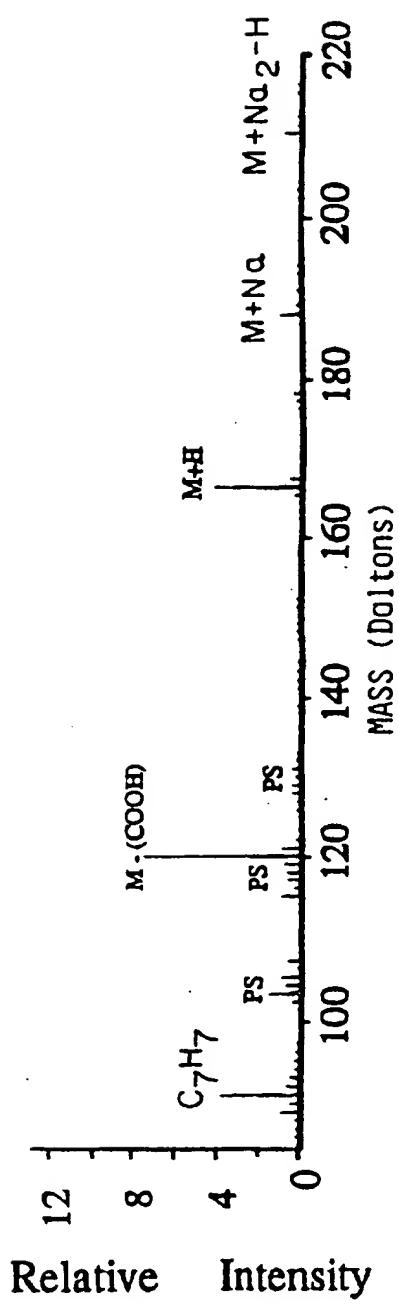
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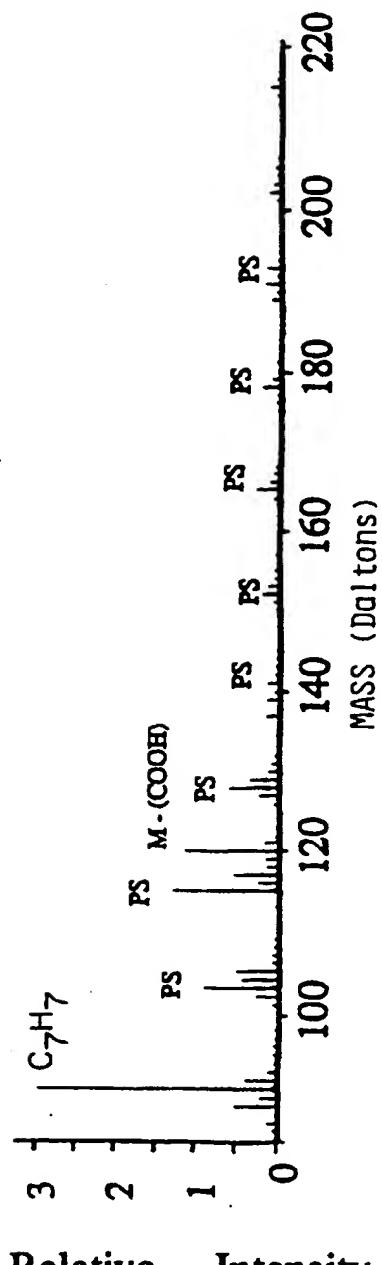
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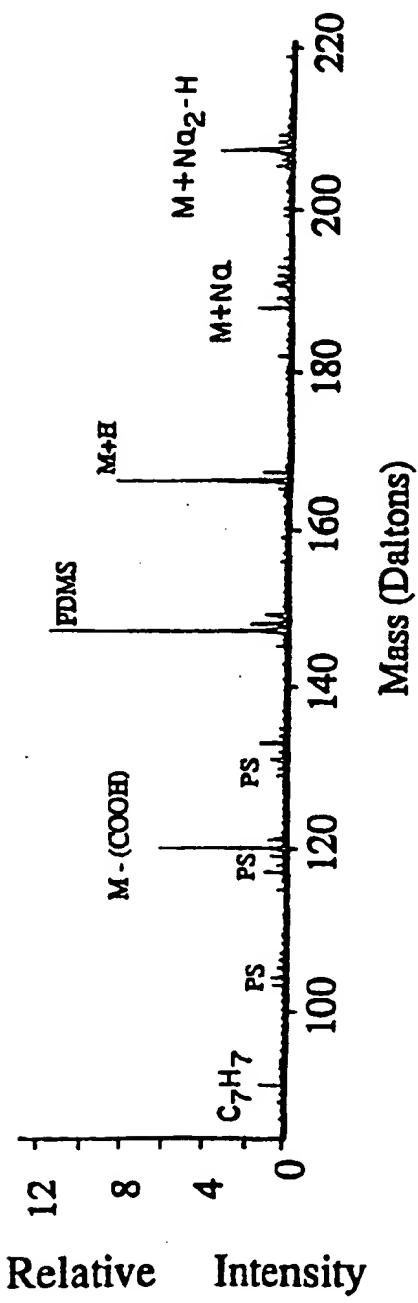
FIG. 2C
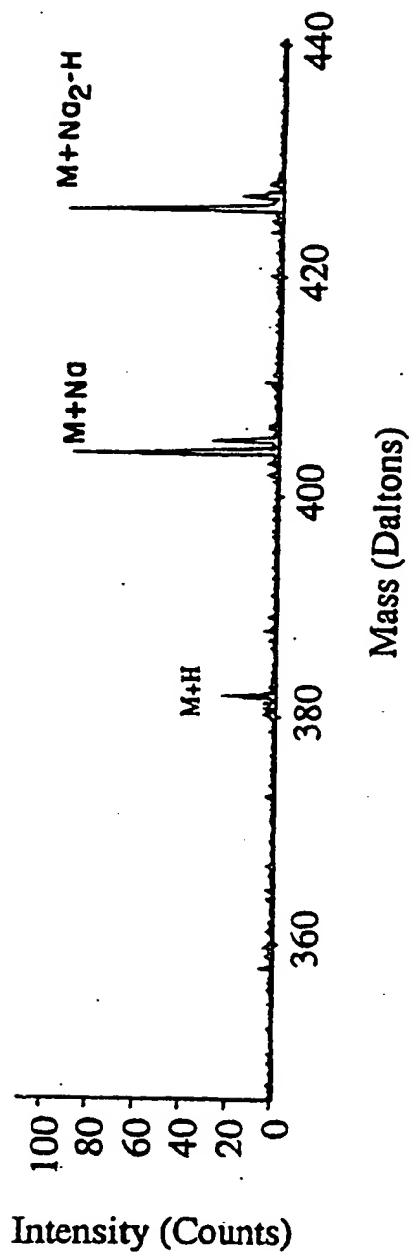
FIG. 3

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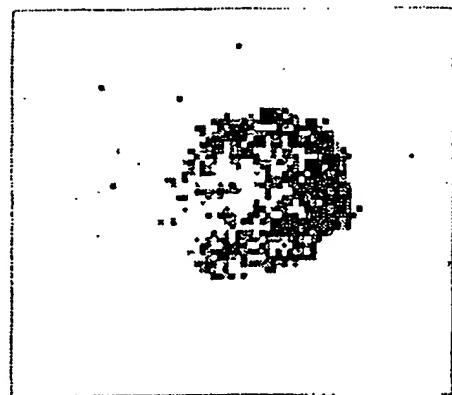
FIG. 4A

Distribution of phenylalanine
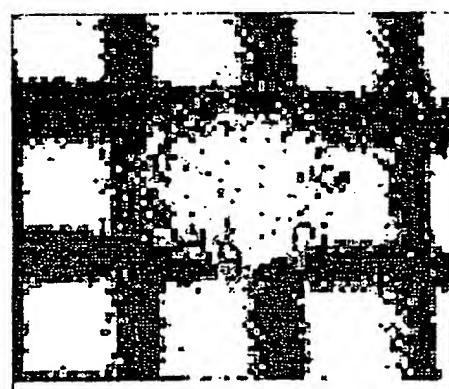
FIG. 4B

Distribution of Cu
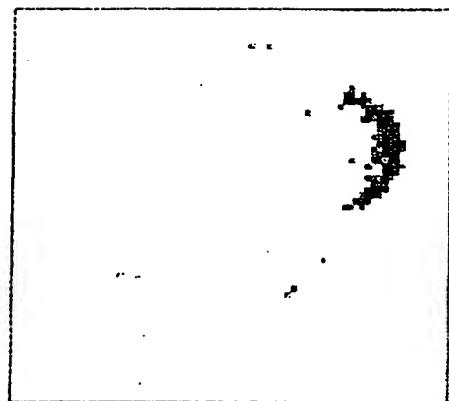
FIG. 5A

Distribution of Leu

FIG. 5B

Distribution of Phe

FIG. 6A

FIG. 6B

Intensity (counts / 100)

Intensity (counts / 100)
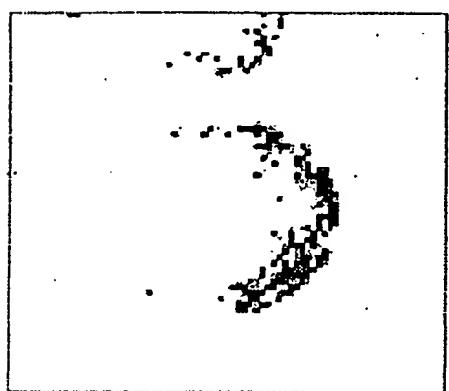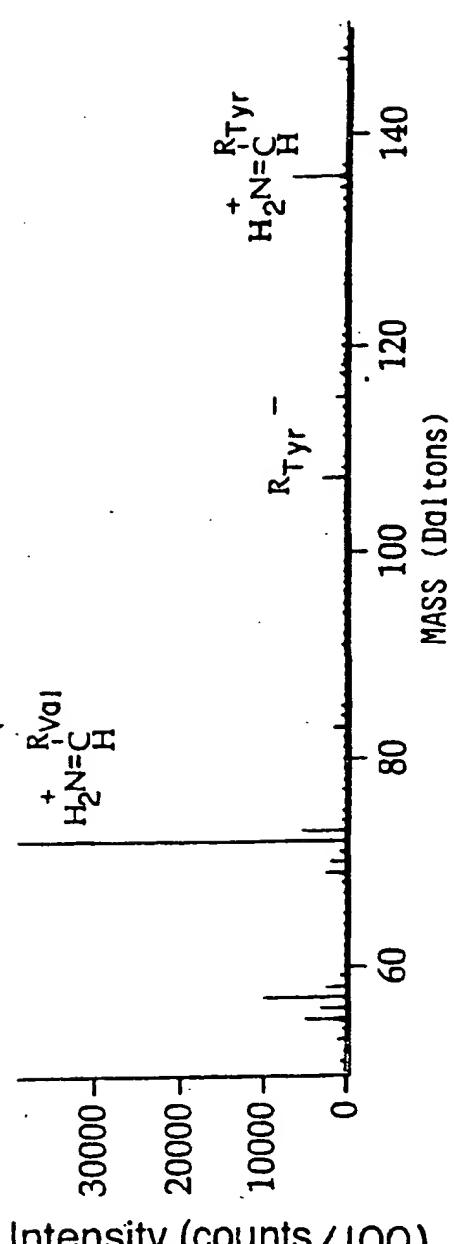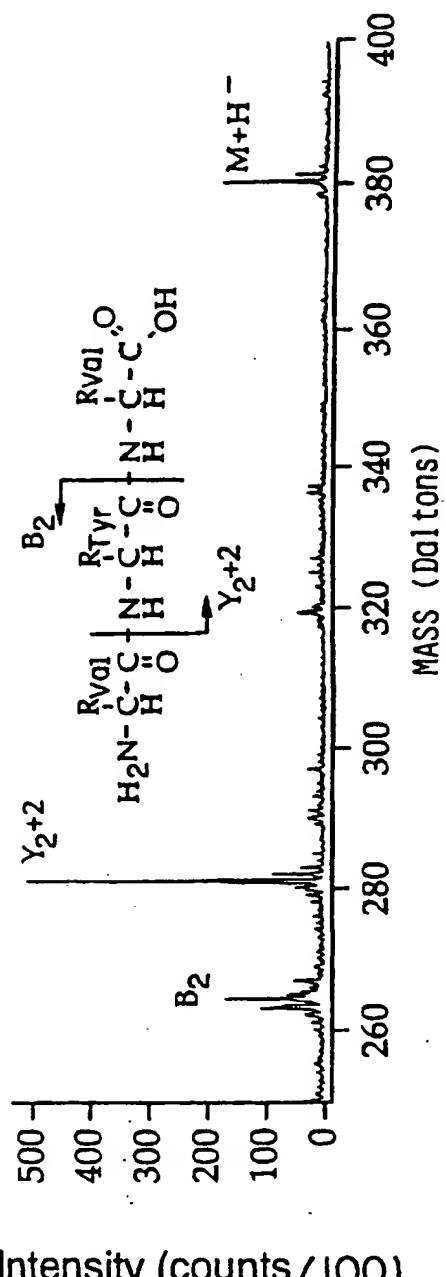
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ANGIOTENSIN ANTAGONIST ON RESINS WITH DIFFERING ACID LABILITY

WANG RESIN-ACID LABILE, SB 218303
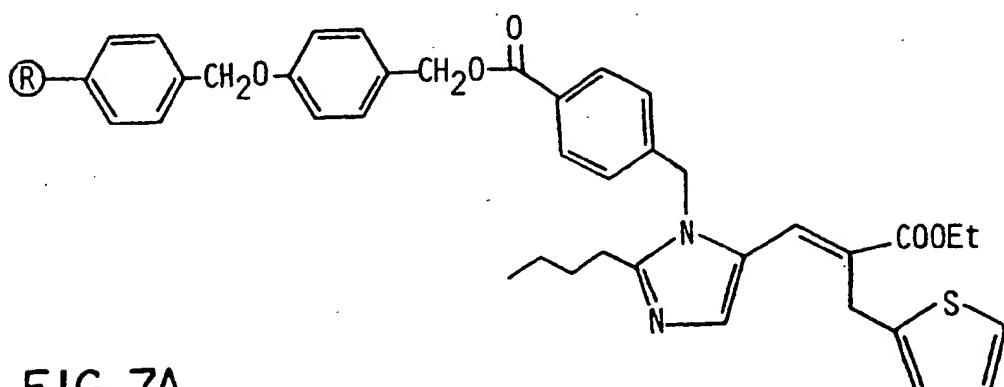
FIG. 7A

NITRO MERRIFIELD RESIN - PHOTO LABILE, SB 220126
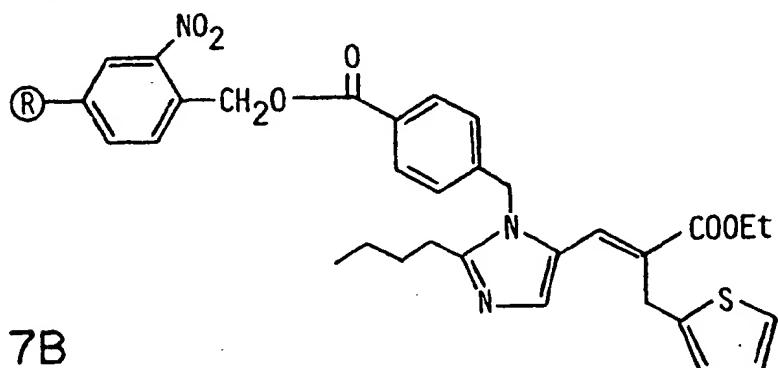
FIG. 7B

THIOACETAL RESIN - ACID LABILE, SB 220127
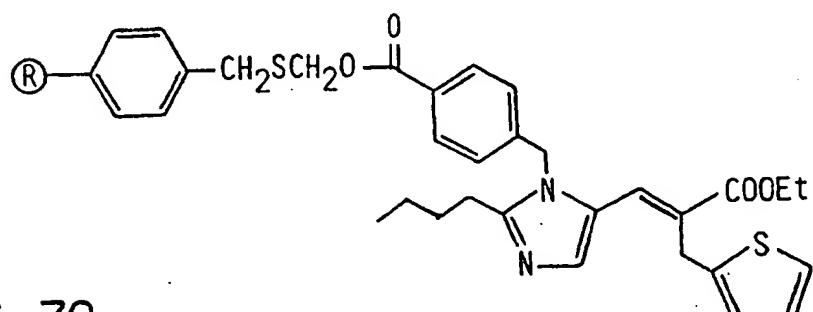
FIG. 7C

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ANGIOTENSIN ANTAGONIST ON RESINS WITH DIFFERING ACID LABILITY

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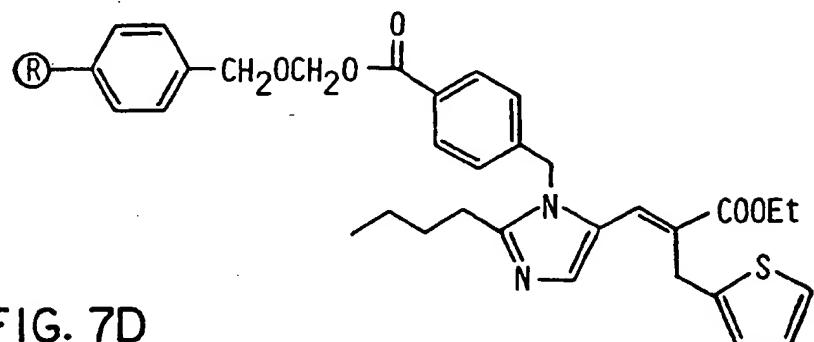
FIG. 7D

SASRIN RESIN, SUPER ACID LABILE, SB 220261

FIG. 7E

FIG. 8A

FIG. 8B

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FIG. 9A

FIG. 9B
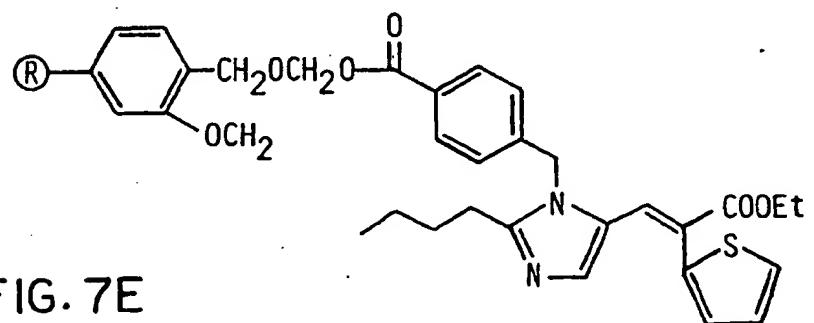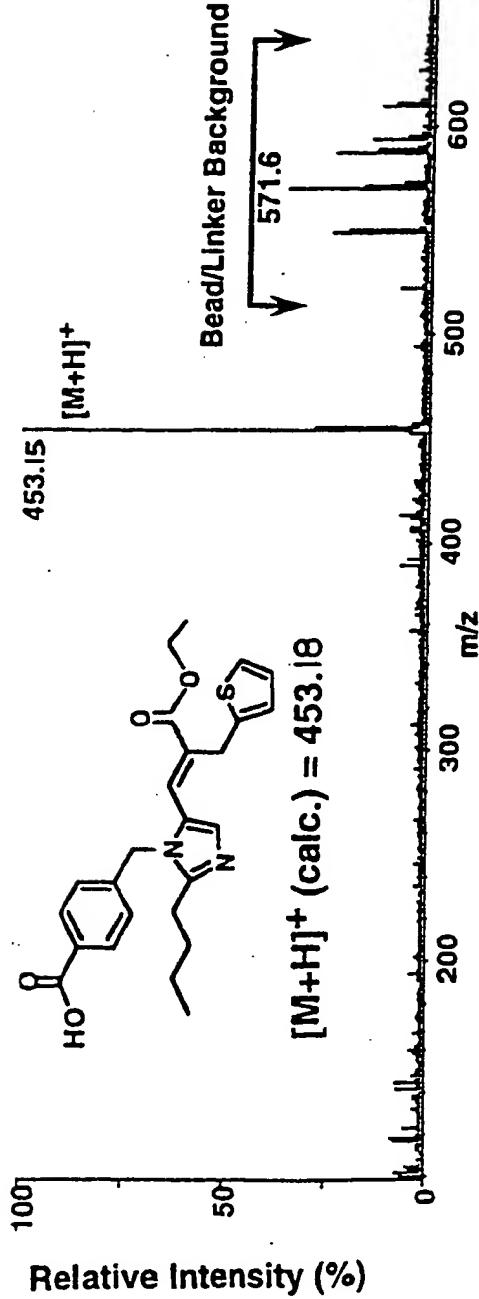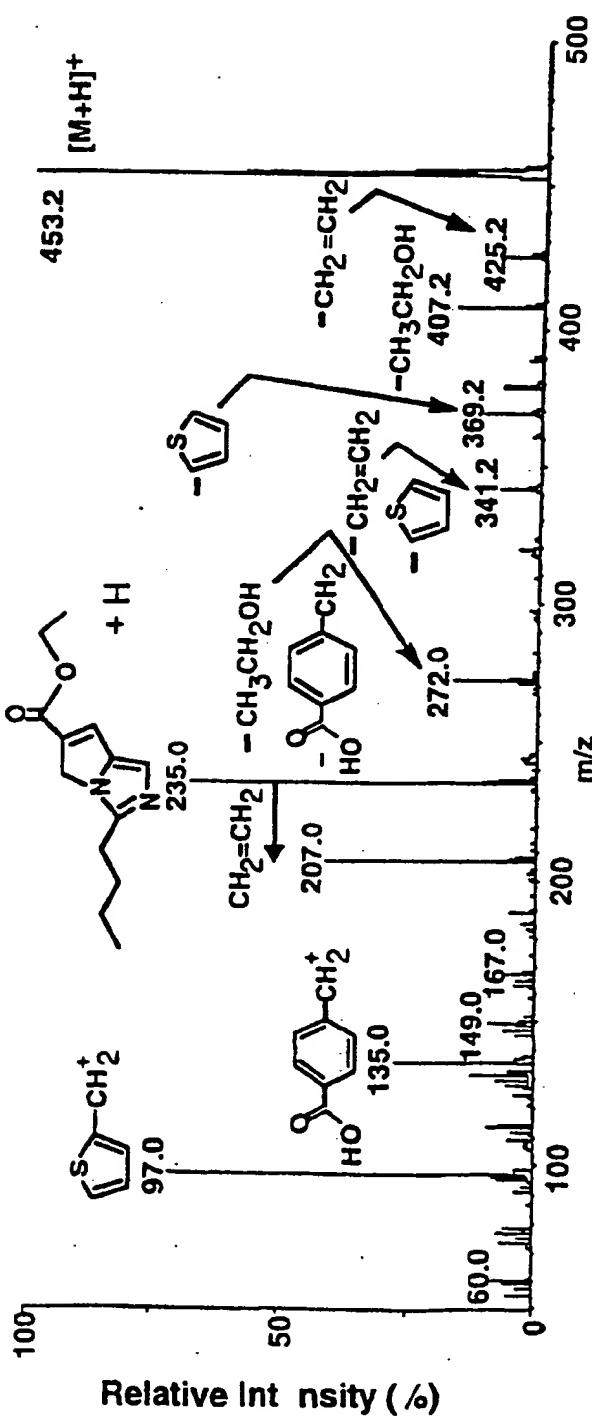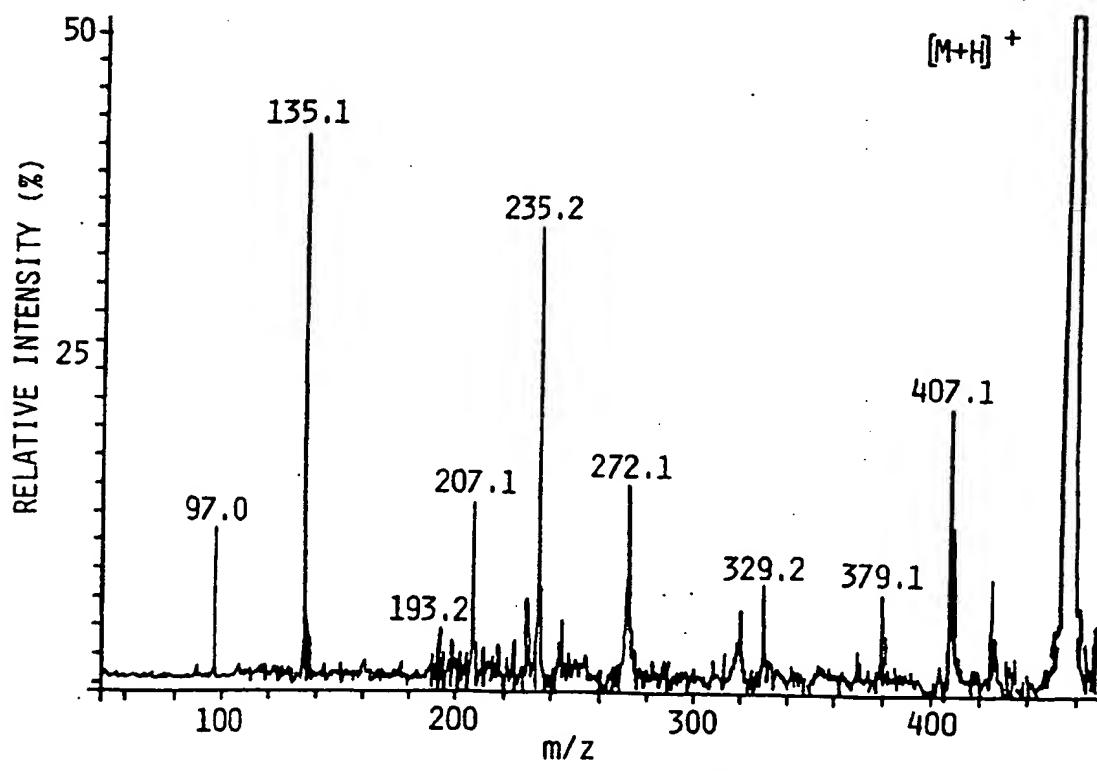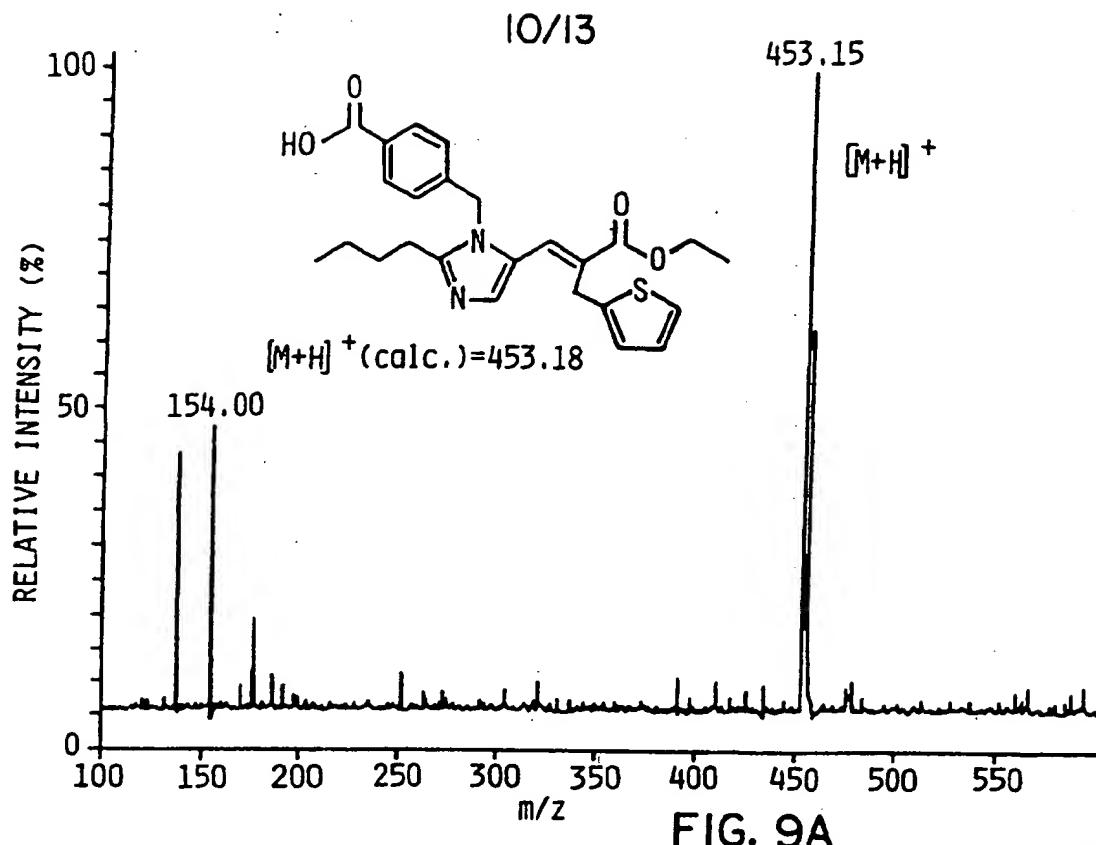
11/13
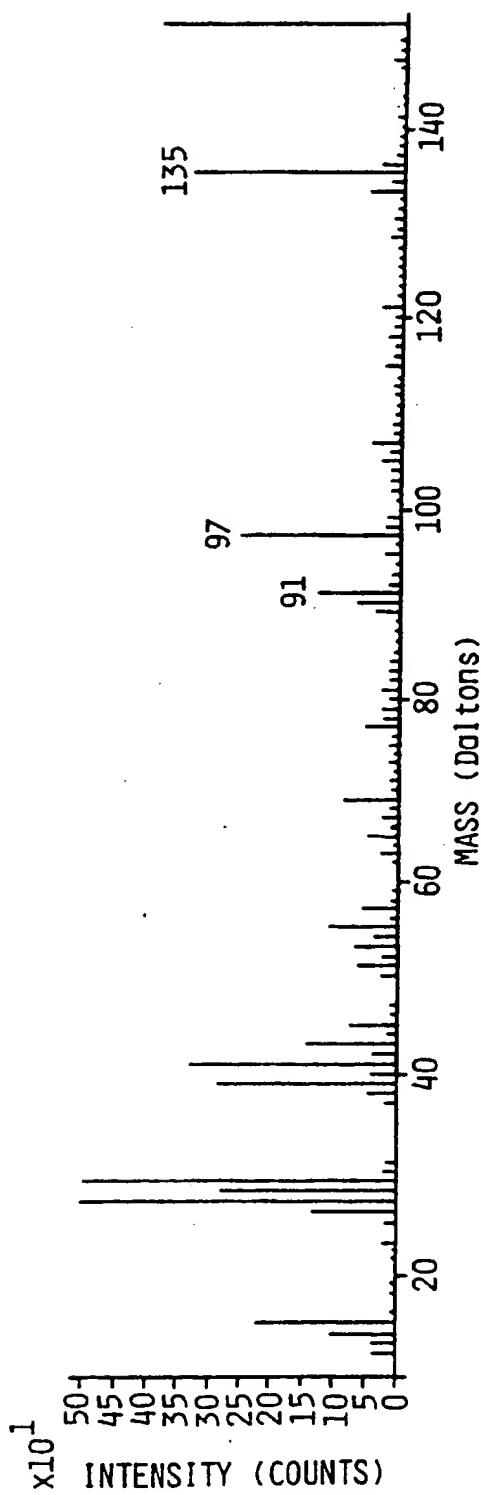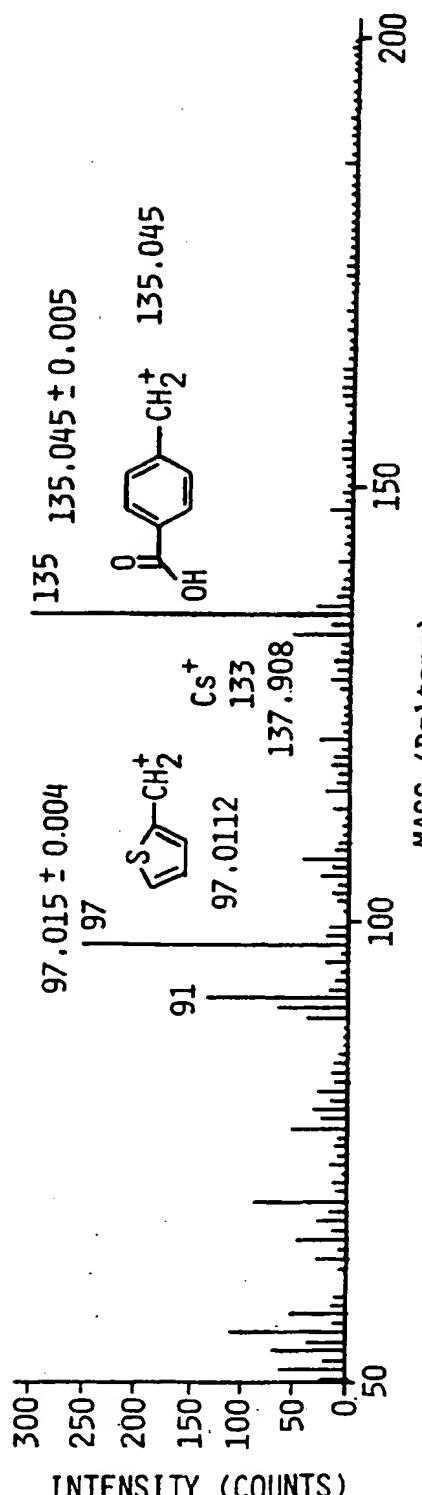
FIG. 10A

FIG. 10B

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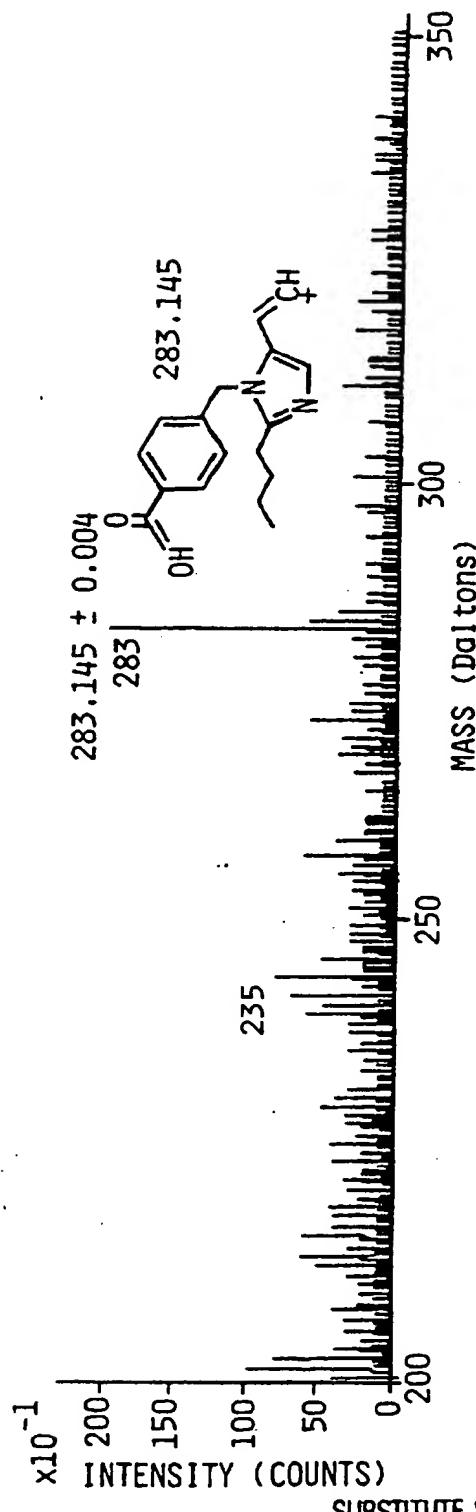
FIG. 10C
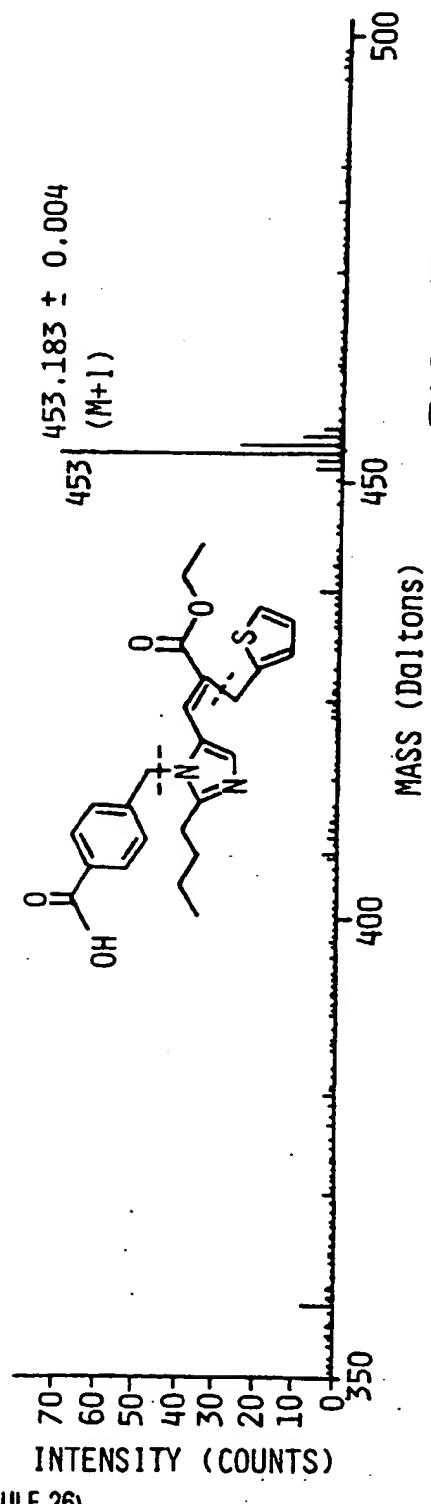
FIG. 10D
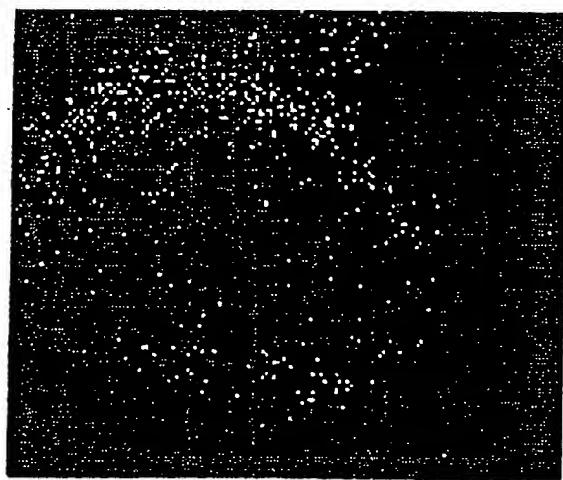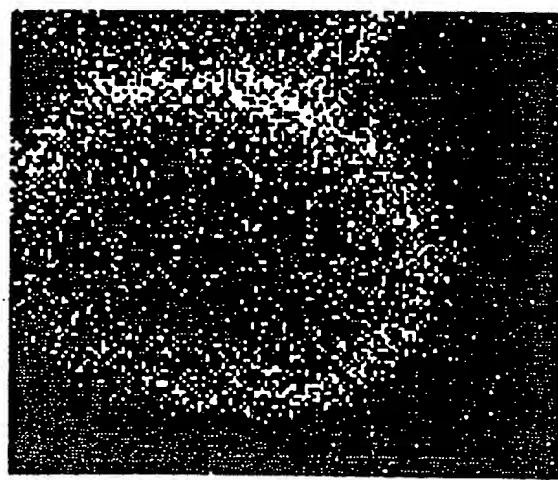
FIG. 11A


FIG. 11B

Inte   nal Application No

PCT/US 95/03355

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C07H21/00   C07K1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07H   C07K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 10, 1992 WASHINGTON US, pages 4505-4509, ZUCKERMANN R.N. ET AL 'Identification of highest-affinity ligands by affinity selection from equimolar peptide mixtures generated by robotic synthesis' see the whole document	1
P,X	SCIENCE, vol. 264, 15 April 1994 LANCASTER, PA US, pages 399-402, BRUMMEL C.L. ET AL 'A Mass Spectrometric Solution to the Address Problem of Combinatorial Libraries' see the whole document	1-20

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 August 1995	1 4. 08. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Day, G

Form PCT/ISA/210 (second sheet) (July 1992)

Inte   onal Application No

PCT/US 95/03355

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,89 10931 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 16 November 1989 see page 19, line 25 - page 24, line 15 ---	1
A	US,A,4 988 879 (ZARE R.N. ET AL) 29 January 1991 see column 12, line 59 - column 13, line 31 ---	1
A	US,A,5 272 338 (WINOGRAD N. AND BENKOVIC S.J.) 21 December 1993 see column 6, line 11 - line 35 -----	1

Intr   nal Application No

PCT/US 95/03355

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8910931	16-11-89	US-A- AU-B- AU-A- EP-A- JP-T- US-A- US-A- US-A-	5010175 645245 3694289 0417157 3505254 5420246 5266684 5225533	23-04-91 13-01-94 29-11-89 20-03-91 14-11-91 30-05-95 30-11-93 06-07-93
US-A-4988879	29-01-91	NONE		
US-A-5272338	21-12-93	NONE		